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## Microbial Biotechnology Providing Bio-based Components for the Food Industry

Laurent Dufossé, Mireille Fouillaud

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The background of the cover features a pattern of overlapping, semi-transparent circular shapes in shades of blue and green, resembling microscopic views of microbial cells or spores. The top half of the cover has a yellow-to-green gradient background, while the bottom half is white.

# **MICROBIAL BIOTECHNOLOGY PROVIDING BIO-BASED COMPONENTS FOR THE FOOD INDUSTRY**

EDITED BY: Laurent Dufossé and Mireille Fouillaud

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# MICROBIAL BIOTECHNOLOGY PROVIDING BIO-BASED COMPONENTS FOR THE FOOD INDUSTRY

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# Editorial: Microbial Biotechnology Providing Bio-based Components for the Food Industry

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**Keywords:** food, ingredient, fermentation, enzyme, peptide, colorant, bacteriocin, thaumatin

## Editorial on the Research Topic

### Microbial Biotechnology Providing Bio-based Components for the Food Industry

This Frontiers Research Topic provides an inter- and multi-disciplinary platform for reviews and researches dedicated to microbial biotechnology providing bio-based components for the food industry. The findings presented in this special issue give a foundation for enlarging the current exploitation of the metabolic diversity in fungi, yeasts, bacteria, and microalgae for improved production of food and other industrial products. Thus, this topic did appeal not just to those interested in the screening and metabolic investigation of microorganisms but also to the industrial biotechnology, the process optimization, the fermentation technology, and the bio-products research community.

Ingredients derived from microbial fermentation or extracted from microalgae are steadily gaining ground in the food industries (Dufossé, 2018). Thickening or gelling agents (e.g., polysaccharides such as xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium glutamate), lipids (polyunsaturated fatty acids—PUFAs, sterols), flavor compounds (gamma-decalactone, diacetyl, methyl-ketones), vitamins, essential amino acids, pigments/colorants (carotenoids, azaphilones) (Dufossé et al., 2014; Venil et al., 2014), surfactants and acidulants (lactic acid, citric acid) are some examples illustrating this trend of the bio-based economy. Efforts have been made and continue to be done in order to reduce the production costs of components produced by algal ponds and microbial fermentation, since synthetic ones or those extracted from natural plant sources can often be produced more economically. Fungi, yeasts, bacteria, and microalgae are considered as promising living organisms for sustainable, large-scale production of commodities such as food, feed, chemicals, materials, and biofuels.

The special issue emphasizes the crucial role that microorganisms and microalgae are currently playing and are likely to continue to play in future as microbial cell factories for the production of food grade components and bio-based ingredients in general. This is due to the versatility in their metabolic pathways and biochemical profiles, amenability for easy large-scale cultivation, and a long history of production by well-investigated production strains. Topics broadly cover studies in Screening and selection, Molecular traits, Metabolic investigation and regulation, Analytical chemistry, Physiology and biochemistry, Process optimization, Fermentation, Extraction techniques, Biomass and bio-products, Cultivation technology, Formulation and applications.

Joseph et al. have reviewed the current state and the perspectives of bioproduction of the recombinant sweet protein thaumatin, which is one of the most promising alternatives for sugar and artificial sweeteners. Recombinant DNA technology is used in the most favorable host known today, the methylotrophic yeast, *Pichia pastoris*.

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Sen et al. have provided comprehensive information about challenges and the way forward for application of microbial pigments in the food industry.

In the same pigments and colorants scientific field, Liu et al. have examined the diversity of chemical structures from *Monascus*, including chemical modification of orange *Monascus* pigments and fine analyses of commercial red and yellow *Monascus* pigments present in Chinese market.

As a global approach Kallscheuer wrote a systematic study about engineered microorganisms for the production of food additives approved by the European Union. Currently, the list of substances authorized by the European Food Safety Authority (EFSA) (referred to as “E numbers”) comprises 316 natural or artificial substances including small organic molecules, metals, salts, but also more complex compounds such as plant extracts and polymers. It is impressive that a broad range of different compounds ranging from small organic acids to more complex secondary metabolites or polymers such as oligopeptides can now be accessed by tailor-made microbial cell factories.

Many articles of this special issue emphasize the power of microorganisms which are able to modify, to improve the properties of many food products or by-products: (i) production from whey of peptides with bacterial antivirulence effects (Ali et al.), (ii) wheat, rice, corn, and amaranth flour proteins treated with microbial transglutaminase, followed by immunoreactivity testing of gluten-sensitized sera toward modified flours (Scarnato et al.), (iii) bioconversion of beet molasses to alpha-galactosidase and ethanol (Álvarez-Cao et al.), (iv) production of fructooligosaccharides from aguamiel, the sap from agave plants (Picazo et al.), or (v) degradation of toxic

steroidal glycoalkaloids from potato juice, a by-product of the potato industry, of the starch processing (Hennessy et al.).

Two papers focused on beneficial effects of microorganisms and microbial metabolites on food preservation. First one on the biopreservative efficacy of *Lactobacillus rhamnosus* bacteriocin on stored fish filets (Sarika et al.) and the second one on the induction of malolactic fermentation of Patagonian Malbec wine with blend cultures of native *Lactobacillus plantarum* and *Oenococcus oeni* strains (Brizuela et al.).

Fine tuning of primary and secondary microbial metabolisms is also of crucial importance, as shown by Liu et al. with small GTPases involved in *Monascus ruber*, a filamentous fungi used for more than a thousand years in Asia for the production food ingredients or as demonstrated by Sgobba et al. who genetically modified *Corynebacterium glutamicum* for utilization of alternative feedstocks such as pentose sugars or hexosamines, to produce the amino acids L-glutamate, L-lysine, and the carotenoid lycopene.

Taken all together, all the papers gathered in this Research Topic illustrate how microbial cell factories (native, engineered, or heterologous) are able to provide at an industrial scale biobased components for the food industry.

Last but not least, we, as Frontiers Guest Editors would like to deeply thanks all the referees for their huge input in handling the manuscripts, and the Frontiers Editorial team.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Engineered Microorganisms for the Production of Food Additives Approved by the European Union—A Systematic Analysis

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In the 1950s, the idea of a single harmonized list of food additives for the European Union arose. Already in 1962, the E-classification system, a robust food safety system intended to protect consumers from possible food-related risks, was introduced. Initially, it was restricted to colorants, but at later stages also preservatives, antioxidants, emulsifiers, stabilizers, thickeners, gelling agents, sweeteners, and flavorings were included. Currently, the list of substances authorized by the European Food Safety Authority (EFSA) (referred to as “E numbers”) comprises 316 natural or artificial substances including small organic molecules, metals, salts, but also more complex compounds such as plant extracts and polymers. Low overall concentrations of such compounds in natural producers due to inherent regulation mechanisms or production processes based on non-regenerative carbon sources led to an increasing interest in establishing more reliable and sustainable production platforms. In this context, microorganisms have received significant attention as alternative sources providing access to these compounds. Scientific advancements in the fields of molecular biology and genetic engineering opened the door toward using engineered microorganisms for overproduction of metabolites of their carbon metabolism such as carboxylic acids and amino acids. In addition, entire pathways, e.g., of plant origin, were functionally introduced into microorganisms, which holds the promise to get access to an even broader range of accessible products. The aim of this review article is to give a systematic overview on current efforts during construction and application of microbial cell factories for the production of food additives listed in the EU “E numbers” catalog. The review is focused on metabolic engineering strategies of industrially relevant production hosts also discussing current bottlenecks in the underlying metabolic pathways and how they can be addressed in the future.

**Keywords:** food additives, E numbers, European Union, microbial production, metabolic engineering, plant natural products

## INTRODUCTION

### History of the Approval of Food Additives in the European Union

In industrial food production, consistent quality of foodstuff, and protection against contamination by harmful microorganisms must be guaranteed. For this purpose, a broad range of natural or artificial food additives are used in today’s food applications, which not only serve for increasing the shelf life of foodstuff, but also for modifying its color, taste, odor, or texture. For preventing

an arbitrary use of potentially harmful substances, there was strong desire toward establishing obligatory guidelines for the use of food additives. The additives sector has played a very important role in the approximation of food law in the European Union (EU). The first regulation, on which the six member states of the European Economic Community (EEC) (Belgium, Germany, France, Italy, Luxembourg, and the Netherlands) were able to agree, was the “Directive on food colorants” in 1962 (Directive 62/2645/EEC), which marks the beginning of a comprehensive harmonization effort. With this directive the E-classification system, a robust food safety system intended to protect consumers from possible food-related risks, was introduced. This classification system lists food additives approved by the EU (or its predecessor organizations) in form of “E numbers” allowing an unambiguous identification of a single compound (which might have several designations, e.g., trivial names), a set of chemically similar compounds or a plant extract.

In 1965, the regulation was extended to preservative substances which may be used in foodstuff (Directive 65/66/EEC). In a similar manner, other classes of food additives were included, e.g., anti-oxidants, emulsifiers, stabilizers, thickeners, and gelling agents in 1978 (Directives 78/663/EEC and 78/664/EEC) and sweeteners in 1994 (Directive 94/35/EC). In parallel, a number of additional intermediate steps in the form of European directives, e.g., for monitoring of purity criteria and for maintenance of national bans on the use of certain additives, was adopted. In 2008, the Framework Regulation (EC) No. 1333/2008 on food additives, and thus directly applicable law, was finally adopted and revised in 2010 in the Directive (EU) No. 257/2010. The principles described therein include health safety, technological necessity and prevention of misleading of food additives. In its current form the list of food additives approved by the EU includes 316 compounds, which are classified according to their major application as colors (E100–E199), preservatives (E200–E299), antioxidants and acidity regulators (E300–E399), thickeners, stabilizers, and emulsifiers (E400–E499), anti-caking agents (E500–E599), flavor enhancers (E600–E699), antibiotics (E700–E799), glazing agents, gases, and sweeteners (E900–E999) and additional additives (E1000–1599). Not all E numbers have been assigned and some E numbers were removed from the list.

## Sources for Getting Access to Food Additives Approved by the EU

The compounds listed in the E numbers catalog comprise small organic molecules, metals, inorganic salts, but also polymers and complex compounds derived from plants or animals (e.g., beeswax, E901). There are in principle three sources of the approved compounds: extraction from the natural producer (e.g., plant material), chemical production or production in (engineered) microorganisms. Not every source is suitable for the production of each of the above-mentioned type of compounds, e.g., metals, inorganic salts, and synthetic colors cannot be produced by microorganisms, whereas complex plant-derived substances (e.g., Gummi arabicum, E414) can often not be produced by chemical synthesis. For this reason, this

review article focuses on the production of natural small organic molecules, which are accessible through metabolic engineering of microorganisms either because they represent natural intermediates of their carbon metabolism or because they can be produced after functional introduction of heterologous pathways (**Table 1**).

In many cases, heterologous enzymes from plants were functionally introduced in microorganisms allowing for the production of natural compounds, which can otherwise only be obtained from plants (Marienhagen and Bott, 2013). A generalized strategy for extraction of natural products from plant material is in most cases not applicable as plants produce such compounds in low amounts and typically harbor complex mixtures of chemically very similar compounds. In addition, the concentrations are subject to regional and seasonal differences and several compounds are only produced under special environmental conditions (e.g., after infections). Although a few plant compounds can nowadays be obtained by direct extraction from plant material (Dai and Mumper, 2010), the above-mentioned drawbacks serve as a strong motivation for reconstructing plant pathways in a controllable and reliable metabolic environment in microbial cell factories (Milke et al., 2018).

The E numbers catalog also includes small organic molecules such as carboxylic acids and aromatic compounds, which are typically produced by the chemical industry. Here, in particular environmental concerns for the production from non-regenerative raw material and the use of toxic chemicals are forces toward establishing alternative production platforms. The fact that microorganisms are naturally capable of producing carboxylic acids and aromatic compounds was recognized as an excellent starting point for metabolic engineering work into this direction in the recent decades (Abbott et al., 2009; Gosset, 2009).

The E numbers catalog classifies food additives according to their major application in foodstuff. However, also distinct and metabolically rather unrelated classes of small organic molecules can have similar application ranges, e.g., aliphatic terpenoids and aromatic phenols are both used as anti-oxidants (**Table 1**). For this reason, compounds mentioned in the following sections are subdivided based on similar biosynthetic pathways or by use of the same precursor metabolites for production.

## METABOLIC ENGINEERING FOR THE MICROBIAL PRODUCTION OF FOOD ADDITIVES

### General Requirements for Microorganisms Used for Production

The underlying metabolic pathways relevant for the production of food additives have significant influence on the choice of the microorganism used for production and on the metabolic engineering strategies followed for establishing and improving production. For some products microorganisms already naturally overproducing the desired compound are tested. In contrast, for products which naturally do not occur in microorganisms it is reasonable to use microbial



**TABLE 1** | Microbially accessible compounds listed in the E numbers catalog sorted by application.

E number	Compound	Compound class
<b>COLORS</b>		
E100	Curcumin	Polyphenol
E101	Riboflavin (vitamin B2)	Nucleotide-derived compound
E160a	Carotenes	Terpenoid
E160b	Bixin, norbixin	Terpenoid
E160c	Capsanthin, capsorubin	Terpenoid
E160d	Lycopene	Terpenoid
E161b	Lutein	Terpenoid
E161g	Canthaxanthin	Terpenoid
E162	Betanin	Polyphenol
E163	Anthocyanins	Polyphenol
<b>PRESERVATIVES</b>		
E210-E213	Benzoic acid and salts	One-ring aromatic
E214-E219	4-Hydroxybenzoic acid esters	One-ring aromatic
E234	Nisin	Oligopeptide
E235	Natamycin	Macrolide
E270	Lactic acid	Carboxylic acid
E296	Malic acid	Dicarboxylic acid
E297	Fumaric acid	Dicarboxylic acid
<b>ANTI-OXIDANTS</b>		
E300-E302	Ascorbic acid and salts	Sugar acid
E307	$\alpha$ -Tocopherol	Terpenoid/phenol
E308	$\beta$ -Tocopherol	Terpenoid/phenol
E308	$\delta$ -Tocopherol	Terpenoid/phenol
E310-E312	Gallic acid esters	One-ring aromatic
E330-E333	Citric acid and salts	Dicarboxylic acid
E334-E337, E354	Tartaric acid and salts	Dicarboxylic acid
E355-E357	Adipic acid and salts	Dicarboxylic acid
E363	Succinic acid	dicarboxylic acid
<b>THICKENERS, STABILIZERS</b>		
E420	Sorbitol	Sugar alcohol
E421	Mannitol	Sugar alcohol
E957	Thaumatin	Protein
E959	Neohesperidin dihydrochalcone	Polyphenol
E967	Xylitol	Sugar alcohol
E968	Erythritol	Sugar alcohol
E422	Glycerol	Short-chain alcohol
<b>ANTI-CAKING AGENTS</b>		
E570	Fatty acids	Carboxylic acid
E574-E579	Gluconic acid and salts	Sugar acid
<b>FLAVOR ENHANCERS</b>		
E620-E625	L-Glutamic acid and salts	Amino acid
E630-E633	Inosinic acid and salts	Nucleotide phosphate
E640	Glycine	Amino acid
<b>GLAZING AGENTS, SWEETENERS</b>		
E920	L-Cysteine	Amino acid
<b>ADDITIONAL ADDITIVES</b>		
E1105	Lysozyme	Protein
E1519	Benzyl alcohol	One-ring aromatic
E1520	1,2-Propanediol	Short-chain alcohol

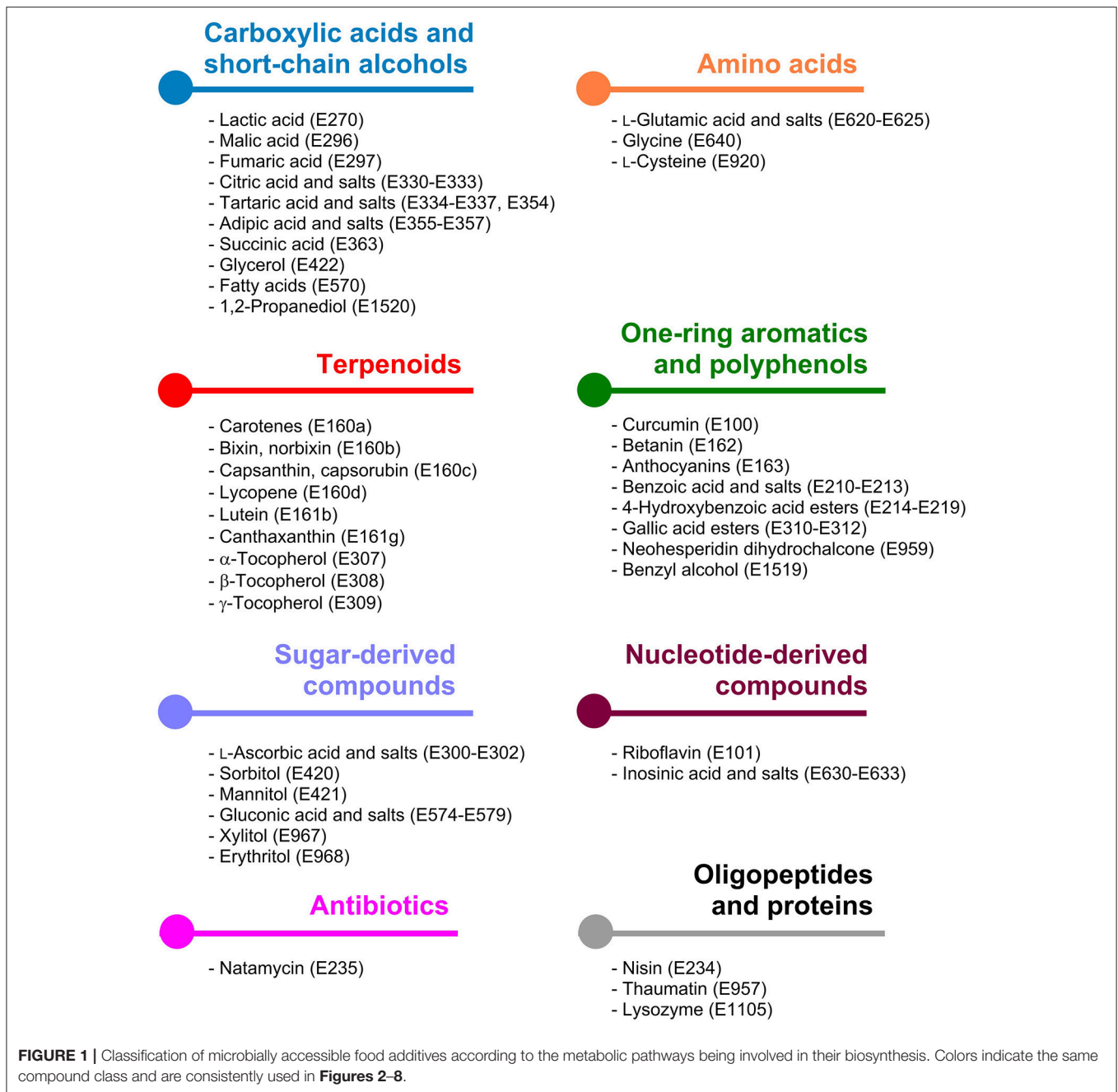
hosts, which can be easily genetically manipulated. General requirements for microbial production strains include fast growth, cultivation in cheap, and defined growth media to high biomass concentrations, non-pathogenicity, and in particular important when food additives are supposed to be produced the obtained product should have GRAS (“generally recognized as safe”) status. In this review article the categories are chosen based on related biosynthetic pathways as shown in **Figure 1** and in each of the following sections the products are sorted by their respective E numbers.

## Carboxylic Acids and Short-Chain Alcohols

Aliphatic carboxylic acids are for the most part used as preservatives or acidity regulators. Most of the carboxylic acids listed in the E numbers catalog can be derived from glycolysis or the tricarboxylic acid (TCA) cycle (**Figure 2**). These comprise lactic acid, citric acid, succinic acid, fumaric acid, and malic acid.

Production of lactic acid (E270) was established in several microorganisms, e.g., lactic acid bacteria (*Lactobacillus* sp. and *Lactococcus* sp.), *Bacillus subtilis*, *Escherichia coli*, *Corynebacterium glutamicum*, yeasts, and microalgae (Abdel-Rahman et al., 2013). In addition to glucose also lignocellulosic biomass, agro-industrial, and food waste or glycerol were used as raw materials. Typically, yields of 0.85–0.98 g lactic acid per g carbon source were obtained. A maximal titer of 225 g/L lactic acid with a yield of 0.99 g/g was e.g., obtained from glucose during fed-batch cultivation of a non-engineered alkaliphilic *Bacillus* species (Meng et al., 2012). In addition to the use as food additive lactic acid is also used in large amounts for the production of the biodegradable polyester polylactic acid (Auras et al., 2011).

The TCA cycle intermediate malic acid (E296) is typically produced in *Aspergillus* species, e.g., *A. flavus*, *A. niger*, and *A. oryzae* (Battat et al., 1991; West, 2011). The latter was further engineered for increased production of malic acid from glycolysis-derived pyruvate by the reductive TCA cycle (**Figure 2**). An engineered strain overexpressing the genes coding for pyruvate carboxylase, malate dehydrogenase, and a C<sub>4</sub>-dicarboxylate transporter was capable of producing 154 g/L malic acid with a yield of 1.02 g/g glucose corresponding to 69% of the maximal theoretical yield (Brown et al., 2013). Similar metabolic engineering strategies were also followed in *S. cerevisiae*, which produced 59 g/L malic acid with a yield of 0.31 g malic acid per g glucose (Zelle et al., 2008). Malic acid production from glycerol was achieved in the plant pathogen *Ustilago trichophora* by combining adaptive laboratory evolution and metabolic engineering approaches (Zambanini et al., 2017). The highest malic acid titer obtained with the optimized strain was 134 g/L. In *E. coli*, the introduction of a mutation into the fumarate reductase gene along with the deletion of genes coding for two malic enzymes and lactate dehydrogenase led to the production of 34 g/L malic acid with a product yield of 1.06 g/g glucose (Zhang et al., 2011b). In very recent studies, additional strategies toward malic acid production in *E. coli* were tested and production from alternative carbon sources such as xylose was demonstrated (Li et al., 2018; Martinez et al., 2018).



For the production of fumaric acid (E297), which is directly formed from malic acid by fumarase activity, strains of *Rhizopus nigricans* or *R. oryzae* are applied as these fungi naturally overproduce fumaric acid. A titer of 85 g/L and yield of 0.85 g fumaric acid per g glucose were obtained during cultivation of *R. oryzae* self-immobilized on plastic discs of a rotary biofilm contactor (Cao et al., 1996). A succinate-producing *E. coli* mutant was turned into a fumarate producer by deletion of three fumarase-encoding genes (Li et al., 2014b). Additional strain engineering toward reducing the production of acetate led to an optimized strain producing 42 g/L fumaric

acid from glycerol with 70% of the maximum theoretical yield.

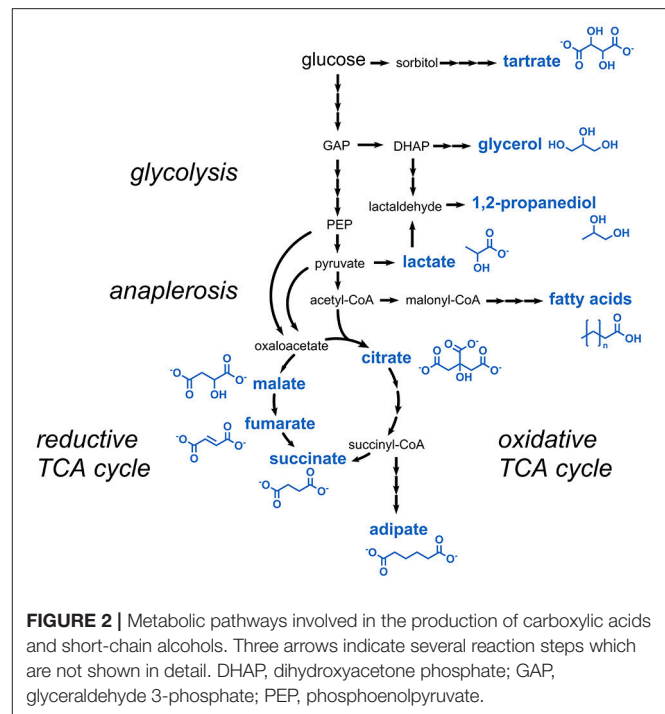
Production of citric acid (E330) at industrial scale is typically conducted based on wild-type *A. niger* (Papagianni, 2007). This yeast naturally overproduces citric acid while the molecular mechanism for its accumulation and secretion is still not entirely understood (Karaffa and Kubicek, 2003). In a very recent study, an industrially used *A. niger* strain obtained from random mutagenesis, which is capable to produce 160 g/L citric acid, was analyzed by transcriptome analysis in comparison to strains producing lower amounts of citric acid (Yin et al., 2017).

Significantly regulated genes coding for enzymes of glycolysis and the TCA cycle as well as for putative citrate transporters are the basis for a detailed analysis of the mechanism for citric acid accumulation and thus for future engineering work. *Yarrowia lipolytica* was recognized as an alternative microorganism for citric acid production from inexpensive carbon sources. Under nitrogen-limiting conditions production of 82 g/L citric acid was shown from glycerol-containing biodiesel waste (Kamzolova et al., 2015).

The dihydroxylated C<sub>4</sub> dicarboxylic acid tartaric acid (E334) is structurally closely related to the TCA cycle intermediates succinic acid and malic acid, and can be converted to oxaloacetic acid in one single dehydration reaction (Hurlbert and Jakoby, 1965). Astonishingly, tartaric acid is not derived from a precursor of the TCA cycle but from a completely unrelated metabolic pathway. In higher plants, it was shown that tartaric acid is produced from L-ascorbic acid via 5-keto D-gluconic acid (DeBolt et al., 2006) (Figure 2). The latter is a natural product obtained from sugar oxidation in the acetic acid bacterium *Gluconobacter oxydans* subsp. *suboxydans*. It was shown that tartaric acid was spontaneously formed from 5-keto D-gluconic acid in culture medium for *G. oxydans* containing vanadium salts (Klasen et al., 1992). A titer of 2.1 g/L tartaric acid from 20 g/L of sorbitol was obtained with this organism (Chandrashekar et al., 1999). However, for commercial applications, large amounts of tartaric acid are obtained today as a by-product from wine industry or by chemical conversion of maleic acid or fumaric acid (Church and Blumberg, 1951; Zhang et al., 2011a).

The C<sub>6</sub> dicarboxylic acid adipic acid (E355) serves as a major building block for the synthesis of nylon polymers, but is also used in foodstuff as acidulant. Adipic acid can be microbially accessed by several pathways, e.g., by the fatty acid synthesis pathway, by degradation of aromatic compounds, or starting from the TCA cycle intermediates succinyl-CoA or 2-oxoglutarate (Polen et al., 2013; Kallscheuer et al., 2017a) (Figure 2). Current efforts for establishing a bio-based production of adipic acid in microorganisms are summarized in a number of recent review articles (Deng et al., 2016; Kallscheuer et al., 2017b; Kruyer and Peralta-Yahya, 2017). The highest published titer for adipic acid of 2.5 g/L was obtained in *E. coli* (Cheong et al., 2016). The constructed strain harbors a heterologous catabolic pathway for adipic acid ultimately leading to succinyl-CoA, which was exploited in the reverse direction for adipic acid biosynthesis. The same pathway is present in the thermophilic bacterium *Thermobifida fusca* in which the overexpression of a gene for an endogenous adipyl-CoA dehydrogenase enabled the production of 2.2 g/L adipic acid without further strain engineering (Deng and Mao, 2015).

The TCA cycle intermediate succinic acid (E363) serves as a flavor enhancer for desserts, dry soups, and drink powder due to its mildly acidic and at the same time slightly salty flavor (Glassner and Datta, 1992). Succinic acid is typically produced via the reductive TCA cycle (Figure 2) under microaerobic or anaerobic conditions for achieving sufficient NADH supply. Several organisms were engineered for its production, e.g., *Actinobacillus succinogenes*, *Basfia succiniciproducens*, *Mannheimia succiniciproducens*, *E. coli*, *C.*



**FIGURE 2** | Metabolic pathways involved in the production of carboxylic acids and short-chain alcohols. Three arrows indicate several reaction steps which are not shown in detail. DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate.

*glutamicum*, and *S. cerevisiae* (Yan et al., 2014; Choi et al., 2016; Meng et al., 2016; Salvachúa et al., 2016; Lange et al., 2017; Mao et al., 2018). The tested strategies during strain engineering included increasing the flux into the pentose phosphate pathway for increasing NADH supply, elimination of competing pathways leading to acetate or lactate formation and overexpression of genes coding for succinate exporter proteins. With these strategies titers in the range of 30–90 g/L and yields of 0.6–1.3 g product per g carbon source were obtained (Ahn et al., 2016).

Nowadays, the triol glycerol (E422) is recognized as a cheap carbon source rather than as a product. This is due to its availability in large quantities as a by-product of biodiesel production (Quispe et al., 2013). However, in the 1990s in particular yeast was extensively engineered for production of glycerol and a maximal titer of 130 g/L and a yield of 0.63 g/g glucose was obtained in the osmotolerant yeast *Candida glycerinogenes* (Wang et al., 2001). In the recent years, there are still several studies reporting on glycerol production, e.g., in the model cyanobacterium *Synechocystis* sp. PCC6803 (Savakis et al., 2015), and also production in yeast strains is still further optimized (Tilloy et al., 2014; Yu et al., 2014; Murashchenko et al., 2016; Semkiv et al., 2017).

The E number E570 lists fatty acids and refers in particular to the long-chain fatty acid stearic acid, which is used as anti-caking agent or as plasticizer in chewing gum. For the production of fatty acids typically oleaginous yeasts such as *Y. lipolytica*, *Trichosporon dermatis*, or *Rhodospiridium toruloides* are employed, which are capable of accumulating long-chain fatty acids with a typical length of 16 or 18 carbon atoms. During fed-batch fermentations lipid contents of 25 to 68% (w/w) with a different composition of long-chain fatty acids were

obtained (Papanikolaou et al., 2002; Li et al., 2007; Huang et al., 2012; Qiao et al., 2015). The key enzyme for further improving fatty acid synthesis is the acetyl-CoA carboxylase, which catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. This enzyme catalyzes the first committed step in the fatty acid synthesis pathways and is strictly regulated by different regulation mechanisms for avoiding fatty acid overproduction (Brownsey et al., 2006; Wei et al., 2016).

The short-chain alcohol 1,2-propanediol (1,2-PD, E1520, also known as propylene glycol) is mainly used as a carrier in the production of flavors for foodstuffs. Several microorganisms were found to be capable of fermenting sugars to 1,2-PD and two metabolic routes for its biosynthesis were identified (Saxena et al., 2010). 1,2-PD can either be derived from the glycolysis intermediate dihydroxyacetone phosphate, which is first dephosphorylated to methylglyoxal and subsequently reduced to lactaldehyde and finally to 1,2-PD (**Figure 2**). Alternatively, in the second pathway lactic acid is reduced to lactaldehyde, which is then further reduced to 1,2-PD. Already in the 1980s, non-engineered *Clostridium thermosaccharolyticum* strains were found to produce 7.9 g/L 1,2-PD with a yield of 0.27 g/g glucose via methylglyoxal (Cameron and Cooney, 1986). Functional introduction of this pathway in an *E. coli* strain optimized for glycerol assimilation allowed for the production of 5.6 g/L 1,2-PD from glycerol (Clomburg and Gonzalez, 2011). The second pathway for 1,2-PD production by degradation of lactic acid was identified in *Lactobacillus buchneri* (Elferink et al., 2001). In *E. coli*, lactaldehyde was identified as a side product from cleavage of L-fucose (Cocks et al., 1974). Lactaldehyde-derived production of 84 mg/L 1,2-PD was reported in a mutant strain of *E. coli* constitutively expressing a gene coding for a propanediol dehydrogenase during growth on L-fucose (Cocks et al., 1974). It turned out that the strain was also capable of utilizing 1,2-PD as sole carbon and energy source (Sridhara et al., 1969). Thus, a functional reversal of the underlying catabolic pathway (e.g., by using anaerobic cultivation conditions) might be the basis for establishing 1,2-PD production *via* lactic acid in *E. coli*.

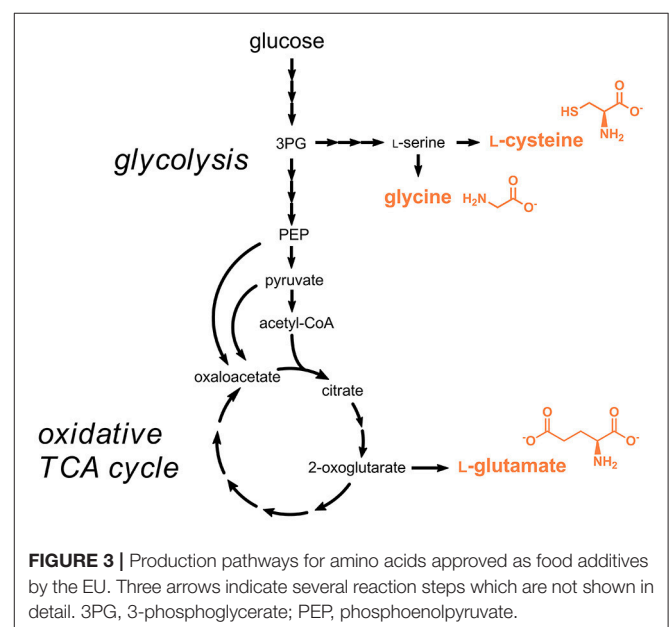
## Amino Acids

Three proteinogenic amino acids are approved by the EU as flavor enhancers, namely L-glutamic acid (E620), glycine (E640), and L-cysteine (E920). L-Glutamic acid and in particular its monosodium salt (E621) are the major compounds responsible for the “umami” taste of foodstuff. L-Cysteine is also used as flour treatment agent to improve baking functionality. All three amino acids can in principle be isolated from hydrolyzed protein or can be produced by engineered microorganisms. In case of glycine, both strategies are not followed for industrial production as this amino acid can be more easily produced by chemical synthesis (Zeng et al., 2016). Especially the expensive step for purification of the L-form from a racemic mixture of chemically synthesized amino acids is not required for glycine as it lacks stereogenic centers.

The L-glutamic acid-overproducing *C. glutamicum* was isolated in Japan in 1956 during a screening campaign for identifying glutamate-producing bacteria (Kinoshita et al., 1957).

In the following decades the mechanism for glutamate secretion was investigated in detail and *C. glutamicum* was extensively engineering toward increased product titers (Hirasawa and Wachi, 2016). Combined activity during strain engineering and optimization of process conditions led to a strain capable of producing 100 g/L L-glutamic acid with a yield of 0.6 g per g glucose (Ault, 2004). Metabolic engineering efforts for increasing L-glutamic acid production in *C. glutamicum* are summarized in several books and review articles (Kimura, 2003; Eggeling and Bott, 2005; Sano, 2009). In recent years, alternative organisms such as *Pantoea ananatis* were also found to be “talented” producers of L-glutamic acid (Katashkina et al., 2009).

L-Cysteine is nowadays for the most part obtained from protein hydrolysis obtained from animal material, e.g., poultry feathers. The natural biosynthesis pathway starting from L-serine and acetyl-CoA was used for engineered L-cysteine production in *E. coli* and *C. glutamicum* (Wada and Takagi, 2006) (**Figure 3**). The rate-limiting reaction in the pathway is the initial step catalyzed by the serine O-acetyltransferase (CysE), which is strictly feedback-inhibited by L-cysteine with enzyme inhibition constants in the micromolar range (Denk and Böck, 1987). Metabolic engineering efforts focused on introducing mutations in the respective gene *cysE* for obtaining less feedback-sensitive enzymes (Kai et al., 2006). By functional introduction of heterologous feedback-insensitive CysE isoenzymes from *Arabidopsis thaliana* into *E. coli* an L-cysteine titer of 1.7 g/L was obtained (Takagi et al., 1999). In a subsequent study, deletion of the gene *yciW*, which encodes an oxidoreductase putatively involved in L-cysteine metabolism in *E. coli*, led to an increased production of L-cysteine, however the titer of 1.7 g/L already obtained in 1999 was not exceeded (Kawano et al., 2015; Takagi and Ohtsu, 2016). Very recently, additional overexpression of the genes coding for SerA (3-phosphoglycerate dehydrogenase), SerB (phosphoserine phosphatase), and SerC (phosphoserine



**FIGURE 3** | Production pathways for amino acids approved as food additives by the EU. Three arrows indicate several reaction steps which are not shown in detail. 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate.



aminotransferase) involved in the synthesis of the precursor L-serine and deletion of genes coding for enzymes involved in the degradation of L-serine and L-cysteine was tested (Liu et al., 2018). The optimized strain produced 5.1 g/L L-cysteine during fed-batch fermentations.

## Terpenoids

Terpenoids (also referred to as isoprenoids) are mostly aliphatic compounds derived from units of isoprene (2-methyl-1,3-butadiene). Most of the terpenoids listed in the E numbers catalog are used as colors and antioxidants. Based on the number of condensed isoprene units (isoprene: C<sub>5</sub>) the products are designated monoterpenes (C<sub>10</sub>), sesquiterpenes (C<sub>15</sub>), diterpenes (C<sub>20</sub>), or tetraterpenes (C<sub>40</sub>). The isoprene units are naturally supplied in form of the two isomers dimethylallyl pyrophosphate (DMAPP) or isopentenyl pyrophosphate (IPP). Two independent pathways responsible for DMAPP/IPP formation were identified: the mevalonate pathway and the methyl-D-erythritol 4-phosphate (MEP) pathway (non-mevalonate pathway) (Goldstein and Brown, 1990; Eisenreich et al., 2004) (Figure 4). The mevalonate pathway produces DMAPP/IPP from three molecules of acetyl-CoA, whereas the MEP pathway requires pyruvate and glyceraldehyde 3-phosphate. The linear, non-cyclic precursor compounds, from which all terpenoids with the corresponding length derive, are geranyl pyrophosphate (C<sub>10</sub>), farnesyl pyrophosphate (C<sub>15</sub>), geranylgeranyl pyrophosphate (C<sub>20</sub>), and phytoene (C<sub>40</sub>). These precursors are cyclized by downstream enzymes and are often further modified, e.g., by hydroxylation reactions.

Lycopene (E160d) is a central tetraterpene, which represents the precursor for biosynthesis of  $\beta$ -carotene (E160a), capsanthin and capsorubin (both E160c), lutein (E161b), and canthaxanthin (E161g) (Figure 4). Before starting metabolic engineering toward lycopene production in *E. coli*, the DMAPP/IPP-forming mevalonate pathway was reconstituted *in vitro* and the steady-state kinetic and biochemical parameters were analyzed (Zhu et al., 2015). Subsequent coupling of the optimized pathway with downstream enzymes required for converting DMAPP/IPP to lycopene led to an *E. coli* strain producing 1.4 g/L lycopene during fed-batch fermentation. Similar titers in the range of 0.9 - 1.1 g/L lycopene were also obtained in related studies performed with *E. coli* as production host (Zhang et al., 2015b; Xu et al., 2018). Alternative hosts for lycopene production include e.g., *Blakeslea trispora* and *Y. lipolytica* (Xu et al., 2007; Matthäus et al., 2014), which are also subject of a recent review article on engineered lycopene production (Hernández-Almanza et al., 2016).

Production of lycopene-derived  $\beta$ -carotene was shown in engineered yeasts such as *S. cerevisiae* and *Rhodotorula glutinis* (Bhosale and Gadre, 2001; Li et al., 2013). In *S. cerevisiae*, genes from the carotenoid-producing yeast *Xanthophyllomyces dendrorhous* were expressed, which led to the production of 5.9 mg  $\beta$ -carotene per g dry weight (Verwaal et al., 2007). One of the introduced genes codes for a 3-hydroxy-3-methylglutaryl-CoA reductase which catalyzes the rate-limiting step in the mevalonate pathway supplying the precursors DMAPP/IPP (Chappell et al., 1995). The entire mevalonate pathway and the  $\beta$ -carotene biosynthetic pathway were also functionally introduced

into *E. coli* (Yoon et al., 2009). In this study, the best-performing strain with plasmid-borne expression of 10 heterologous genes produced 0.47 g/L  $\beta$ -carotene in complex medium with 2% glycerol.

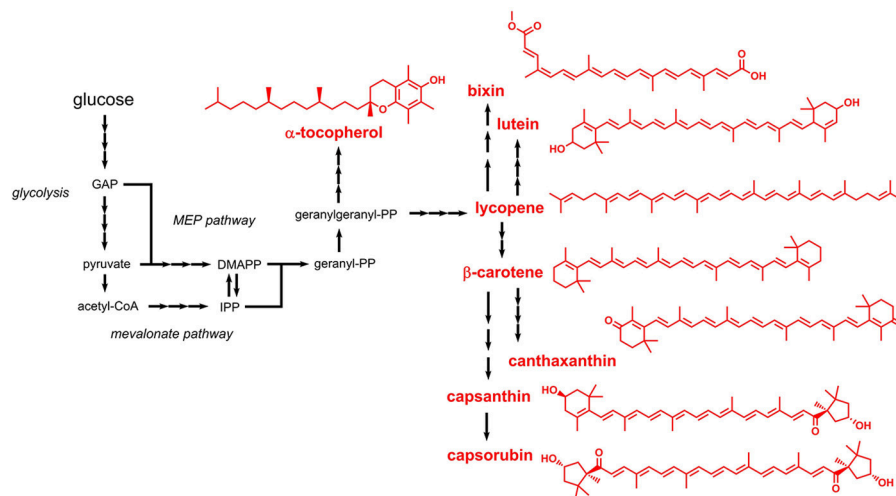
Apocarotenoids such as bixin and norbixin (E160b) are obtained by oxidative cleavage of lycopene (Figure 4). Both compounds were produced by functional introduction of the lycopene cleavage dioxygenase from the plant achiote (*Bixa orellana*) together with an aldehyde dehydrogenase and a carboxyl methyltransferase into an engineered lycopene-producing *E. coli* strain (Bouvier et al., 2003). A production level of 5 mg bixin/g dry weight was obtained.

Capsanthin and capsorubin (E160c) belong to the class of xanthophylls (oxygen-containing carotenoids) and are the major carotenoids in red pepper fruits (Lefebvre et al., 1998). Both compounds can be produced from lycopene by six reactions steps including two cyclization and two hydroxylation steps and subsequent epoxidation and de-epoxidation. Production of capsanthin and capsorubin has not been achieved in microorganisms until today, but strategies for production in *E. coli* were proposed (Misawa, 2013).

Lutein (E161b), which is also a plant-derived xanthophyll, is produced from lycopene by two cyclization and two hydroxylation reactions (Kim and DellaPenna, 2006). Biotechnological lutein production focused exclusively on the use of microalgae, which naturally produce lutein (Fernández-Sevilla et al., 2010). It turned out that lutein production capabilities strongly depend on environmental and operating factors. A third xanthophyll, canthaxanthin (E161g), is naturally produced from  $\beta$ -carotene by two reaction steps, which are both catalyzed by the  $\beta$ -carotene ketolase CrtW. Several microorganisms naturally producing canthaxanthin were identified, e.g., *Haloferax alexandrinus*, *Gordonia jacobaea*, and *Dietzia natronolimnaea* (de Miguel et al., 2000; Asker and Ohta, 2002; Gharibzahedi et al., 2012). With mutated strains and by choosing cultivation conditions promoting canthaxanthin production, product titers in the range of 2–8 mg/L were obtained (Gharibzahedi et al., 2012; Rostami et al., 2014). The  $\beta$ -carotene ketolase gene *crtW* from *Agrobacterium aurantiacum* was already functionally expressed in an *E. coli* strain engineered for  $\beta$ -carotene production, which led to the production of detectable amounts of canthaxanthin in this organism (Misawa et al., 1995).

Some carotenoid biosynthetic pathways lead to the production of aromatic compounds, e.g., okenone and isorenieratene (these compounds are not listed in the E numbers catalog). In such cases the aliphatic rings, e.g., in  $\beta$ -carotene, are oxidized to aromatic (benzene) rings. Tocopherols (E307, E308, and E309) also contain an aromatic ring but represent an exception to the above-mentioned biosynthesis strategy. Tocopherols can be classified as terpenoids because they are derived from geranylgeranyl pyrophosphate (Figure 4). However, the aromatic ring present in tocopherol is not formed during terpenoid biosynthesis but is obtained from homogentisate (2,5-dihydroxyphenylacetate), which in turn is produced from the aromatic amino acid L-tyrosine (Arias-Barrau et al., 2004). Thus, microbial production of tocopherol requires engineering of two unrelated pathways, namely the





**FIGURE 4** | Biosynthetic pathways leading to terpenoids approved as food additives by the EU. Three arrows indicate several reaction steps which are not shown in detail. GAP, glyceraldehyde 3-phosphate; MEP, methyl erythritol phosphate; DMAPP, dimethylallylpyrophosphate; IPP, isopentenylpyrophosphate.

terpenoid biosynthetic pathway and the L-tyrosine-forming shikimate pathway. This might be a reason why tocopherol production focused on natural producers, namely photosynthetic microorganisms such as *Dunaliella tertiolecta* and *Euglena gracilis* (Tani and Tsumura, 1989; Carballo-Cárdenas et al., 2003). In *E. gracilis*, a titer of 144 mg/L  $\alpha$ -tocopherol was achieved.

## One-Ring Aromatics and Polyphenols

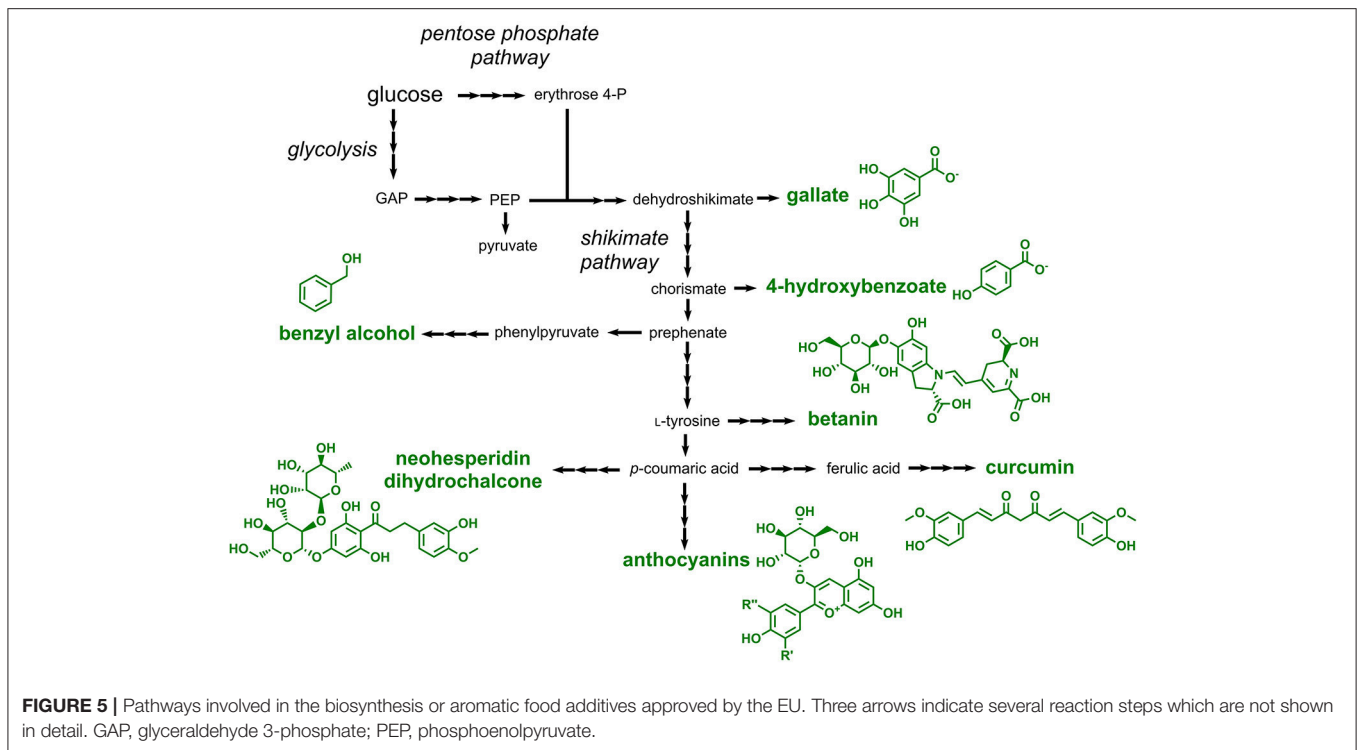
Aromatic compounds approved as food additives by the EU can be classified into two large groups: one-ring aromatics and polyphenols. One-ring aromatics are typically benzoic acid derivatives, while polyphenols comprise a large group of structurally diverse and more complex secondary metabolites in plants (Quideau et al., 2011). All compounds of these two classes are derived from the aromatic amino acid-forming shikimate pathway, which requires the pentose phosphate pathway-derived erythrose 4-phosphate and glycolysis-derived phosphoenolpyruvate as precursors (Figure 5). One-ring aromatics are typically produced from intermediates of the shikimate pathway (e.g., shikimate or chorismate), whereas all polyphenols are derived from phenylpropanoids, which in turn are produced by deamination of the aromatic amino acids L-phenylalanine or L-tyrosine (Vogt, 2010) (Figure 5).

Four different one-ring aromatics, which can be accessed with microorganisms, are listed as E numbers: benzoic acid and different salts derived thereof (E210–E213), 4-hydroxybenzoic acid esters (E214–E219), gallic acid esters (E310–E312), and benzyl alcohol (E1519). Although a natural pathway for the production of benzoic acid by chain-shortening of the phenylpropanoid cinnamic acid was identified in plants (Moerkercke et al., 2009), this compound was not produced in engineered microorganisms until today. This is probably due to the fact that benzoic acid can be easily obtained from petroleum-derived toluene (Kaeding et al., 1965). In contrast,

several microorganisms were engineered for the production of 4-hydroxybenzoic acid and gallic acid (3,4,5-trihydroxybenzoic acid), which are direct precursors for the corresponding methyl-, ethyl- and propyl esters approved as food additives. 4-hydroxybenzoic acid is microbially produced by cleavage of the shikimate pathway intermediate chorismate (Siebert et al., 1994) (Figure 5). In addition to the functional introduction of the required chorismate pyruvate lyase the feedback-regulation at the initial step of the shikimate pathway needs to be abolished. For this purpose, feedback-resistant enzymes catalyzing this initial step are applied (Weaver and Herrmann, 1990; Fukuda et al., 1991; Jossek et al., 2001). By following this strategy, 4-hydroxybenzoic acid was produced e.g., in *E. coli*, *C. glutamicum*, *S. cerevisiae*, and *Pseudomonas putida* (Meijnen et al., 2011; Noda et al., 2016; Aversch et al., 2017; Kallscheuer and Marienhagen, 2018). Very recently, a titer of 36.6 g/L and a yield of 0.31 g/g glucose was obtained in engineered *C. glutamicum* during fed-batch fermentation (Kitade et al., 2018). An *E. coli* strain overproducing the shikimate pathway intermediate 3-dehydroshikimate was capable of producing 20 g/L gallic acid, when an endogenous gene coding for a dehydroshikimate dehydrogenase was overexpressed (Kambourakis et al., 2000). 114 mg/L benzyl alcohol was produced from glucose in *E. coli* using a non-natural pathway starting from the shikimate pathway intermediate phenylpyruvate (Pugh et al., 2015).

Plant-derived polyphenols present in fruits and vegetables are part of our daily diet. Thus, it is surprising that only four polyphenols are approved as food additives by the EU. These include curcumin (E100, a yellow colorant), betanin (E162, a red colorant), anthocyanins (E163, a large class of plant colorants), and neohesperidin dihydrochalcone (E959, a natural sweetener).

Curcumin is produced starting from the phenylpropanoid *p*-coumaric acid, which in turn is obtained from the non-oxidative deamination of L-tyrosine (Katsuyama et al., 2009). *p*-Coumaric acid is first converted to the phenylpropanoid ferulic acid by



hydroxylation and subsequent *O*-methylation. Ferulic acid is then CoA-activated by the activity of a CoA-ligase. The key enzyme curcumin synthase subsequently condenses the CoA-thioesters of ferulic acid and of a diketide derived from ferulic acid yielding curcumin. Curcumin production in the range of 60 mg/L from supplemented ferulic acid was demonstrated in *E. coli* by expression of heterologous genes coding for the CoA ligase and the curcumin synthase (Katsuyama et al., 2008). In a more recent study, curcumin production with a titer of 0.6 mg/L was achieved in *E. coli* starting from L-tyrosine (Wang et al., 2015).

Betanin (E162) is a glycosylated betacyanine, which is a major compound present in beetroot. This natural red dye is produced starting from L-tyrosine, which is first hydroxylated yielding L-3,4-dihydroxyphenylalanine (also known as Levodopa or L-DOPA). By the activity of two independent enzymes one molecule of L-DOPA is converted to cyclo-DOPA (precursor 1) and a second one to betalamic acid (precursor 2). Precursor 1 and 2 are subsequently ligated yielding betanidin, which is subsequently 5-*O*-glycosylated to betanin (Tanaka et al., 2008). The entire pathway was recently reconstructed in *S. cerevisiae* by functional expression of genes from various plants (Grewal et al., 2018). The constructed strains were not only capable of producing 17 mg/L betanin, but also converted alternative aromatic amines (instead of cyclo-DOPA) to the corresponding betacyanine dyes.

In addition to betanin, also plant-derived anthocyanins (E163) find an application as colorants in foodstuff. For the synthesis of anthocyanins the L-tyrosine-derived and CoA-activated phenylpropanoid *p*-coumaroyl-CoA acid is first condensed with three molecules of malonyl-CoA yielding a

tetraketide intermediate, which is cyclized to the compound naringenin chalcone. All steps during this reaction, the malonyl-CoA-dependent chain elongation as well as the cyclization step, are catalyzed by chalcone synthases (Ferrer et al., 1999). By the activity of chalcone isomerases, naringenin chalcone is subsequently isomerized to the flavanone naringenin. Naringenin, the first compound in the pathway harboring the typical flavonoid core structure, is then further converted by plant dioxygenases and reductases giving rise to the anthocyanidin pelargonidin, which is further stabilized by *O*-glycosylation at C3 (the glycosylated anthocyanidin is then designated anthocyanin). Taken together, the overall pathway from *p*-coumaric acid includes seven reaction steps (1: CoA activation, 2: chain elongation and cyclization, 3: isomerization, 4: hydroxylation, 5: reduction, 6: oxidation, 7: glycosylation) (Figure 5). The resulting pelargonidin-3-*O*-glucoside can be further converted to related anthocyanins by ring hydroxylation or *O*-methylation reactions. For achieving microbial anthocyanin production in particular *E. coli* strains were constructed (Yan et al., 2005, 2008; Lim et al., 2015). Recently, the production of 9.5 mg/L pelargonidin-3-*O*-glucoside (callistephin) from glucose was achieved by a co-culture of four different *E. coli* strains together expressing 15 heterologous genes (Jones et al., 2017).

Neohesperidin dihydrochalcone (E959) is a rutinosylated chalcone, i.e., it is decorated with rutinose (rhamnose- $\alpha$ -1,6-glucose) (Figure 5). This chalcone is present in citrus fruits and is applied as a natural sweetener in beverages, yogurt and ice cream. Neohesperidin dihydrochalcone was not produced in engineered microbes so far, but a very similar compound, naringin dihydrochalcone, which differs

from neohesperidin dihydrochalcone only by one *O*-methyl-group, was obtained in engineered *S. cerevisiae* (Eichenberger et al., 2017). The constructed strain heterologously expressed nine genes and produced 12 mg/L naringin dihydrochalcone from glucose. Based on these results a microbial production of neohesperidin dihydrochalcone can be achieved by including an additional *O*-methyltransferase capable of converting naringin dihydrochalcone to neohesperidin dihydrochalcone.

## Sugar-Derived Compounds

Subject of this section are food additives listed in the E numbers catalog, which are more or less directly derived from the sugar metabolism. Many different compounds can in principle be “derived from sugar”; hence only compounds derived from glycolysis and the pentose phosphate pathway are included here (Figure 6).

L-Ascorbic acid (E300, also known as vitamin C) is a vitamin traditionally consumed with fruits and vegetables, which is today produced at the scale of more than 100,000 tons per year (Pappenberger and Hohmann, 2013). L-Ascorbic acid is also extensively added to foods, beverages and pharmaceuticals. The established industrial production process of L-ascorbic acid is based on D-sorbitol or sorbose, which are first oxidized to 2-keto-L-gulonic acid by *Gluconobacter oxydans* or *Ketogulonicigenium vulgare*, respectively and then chemically converted to L-ascorbic acid (Pappenberger and Hohmann, 2013). *G. oxydans* was capable to produce 130 g/L 2-keto-L-gulonic acid from 150 g/L sorbitol (Saito et al., 1997), while a titer of 60 g/L was achieved from 70 g/L sorbose in *K. vulgare* (Ning et al., 1988).

Sorbitol (E420), the precursor for industrial L-ascorbic acid production, is also a food additive approved by the EU and is mainly used as natural sweetener, e.g., in chewing gum. Although sorbitol is traditionally synthesized by catalytic hydrogenation of glucose (Kusserow et al., 2003), it was also produced in engineered microorganisms from glucose in a single reduction step. In *Zymomonas mobilis* the enzyme glucose-fructose oxidoreductase was shown to catalyze the reduction of glucose to sorbitol by simultaneously oxidizing fructose to gluconolactone (Zachariou and Scopes, 1986). Not surprisingly, this organism was exploited for the production of sorbitol, in particular in the 1990s (Silveira and Jonas, 2002). In more recent studies also alternative hosts were applied, e.g., the cyanobacterium *Synechocystis* sp. and the lactic acid bacterium *Lactobacillus plantarum* (Jan et al., 2017; Chin et al., 2018).

Additional polyols with applications similar to sorbitol comprise mannitol (E421), xylitol (E967), and erythritol (E968) (Figure 6). These are also produced industrially by catalytic hydrogenation (Schiweck et al., 2000), but several microorganisms were engineered toward their production. Mannitol is typically produced in lactic acid bacteria, e.g., *Lactococcus lactis* or *Lactobacillus reuteri* via the glycolysis intermediate fructose-6-phosphate, which is reduced to mannitol-1-phosphate and subsequently dephosphorylated. Yields of 0.5–0.6 g mannitol per g glucose or fructose were obtained (Song and Vieille, 2009). A constructed *C. glutamicum* strain expressing genes coding for a mannitol dehydrogenase from *Leuconostoc pseudomesenteroides* and a glucose/fructose

transporter from *Z. mobilis* was capable to produce 87 g/L mannitol from 94 g/L fructose (Bäumchen and Bringer-Meyer, 2007). The xylose reductase of *Pichia stipitis* was introduced into *S. cerevisiae*, which led to a xylose conversion rate of 95% in the recombinant strain (Hallborn et al., 1991). In a more recent study, the sugar import of a *Kluyveromyces marxianus* strain harboring a xylose reductase from *Neurospora crassa* was engineered and a final titer of 312 g/L xylitol was obtained during fed-batch fermentation (Zhang et al., 2015a).

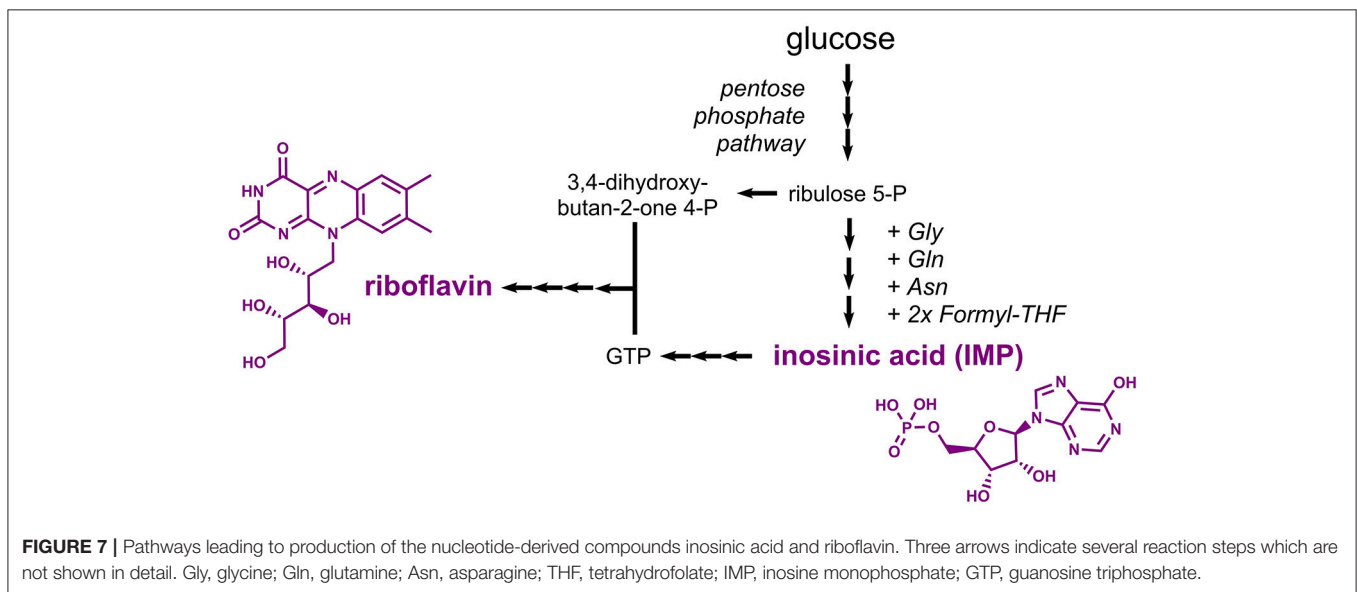
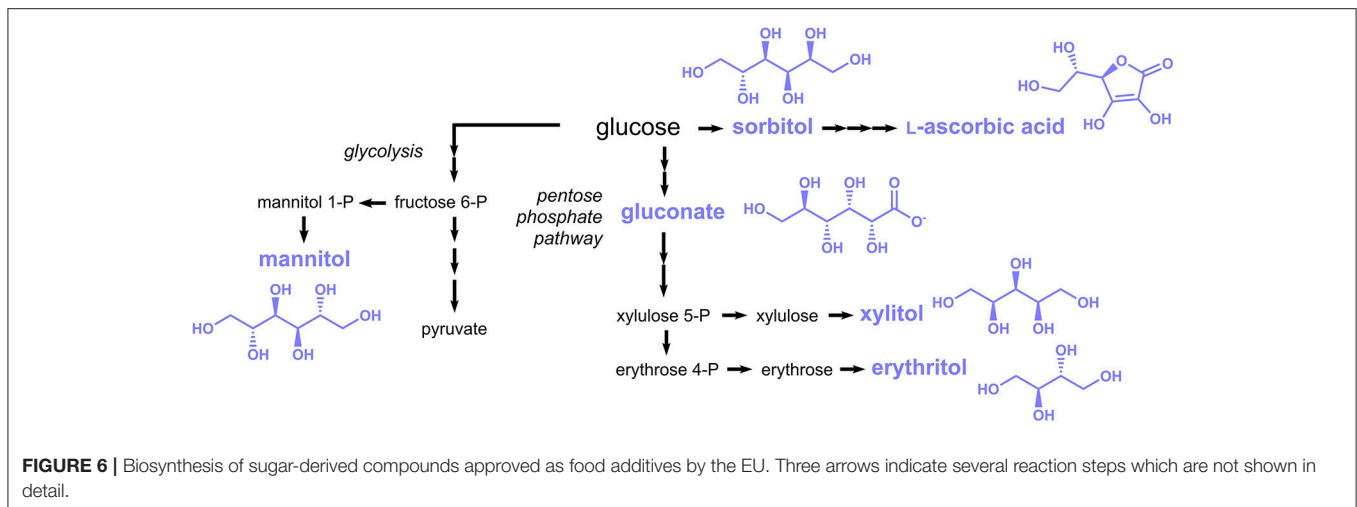
For the production of erythritol, erythrose 4-phosphate (E4P) derived from the pentose phosphate pathway is first dephosphorylated to erythrose and subsequently reduced to erythritol (Figure 6). In some microorganisms, e.g., lactic acid bacteria, E4P is first reduced to erythritol 4-phosphate and then dephosphorylated. Yields of 0.3 to 0.4 g/g glucose and product titers in the range of 120–250 g/L were obtained in microorganisms engineered toward erythritol production (Moon et al., 2010).

Oxidation of glucose at the C1-atom leads to gluconic acid (E574) (Figure 6), which is used in food applications as an acidity regulator. The enzymatically-driven oxidation reaction can either be coupled to the reduction of a cofactor, e.g., NAD(P)<sup>+</sup>, FAD or a quinone (glucose 1-dehydrogenase) or alternatively molecular oxygen can serve as oxidant (glucose oxidase). The latter reaction also yields H<sub>2</sub>O<sub>2</sub> as byproduct, which needs to be rapidly detoxified by a catalase. Production of gluconic acid was achieved in an *A. niger* strain with high activity of glucose oxidase and catalase (Znad et al., 2004). After 60 h of cultivation a nearly complete conversion of 150 g/L glucose to 150 g/L gluconic acid was observed. Further optimization of the process conditions reduced the production time to 15 h and improved the yield and final titer to 1.05 g/g and 311 g/L respectively (Lu et al., 2015).

## Nucleotide-Derived Compounds

Riboflavin (E101, vitamin B<sub>2</sub>), a yellow colorant, and inosinic acid (E630), a flavor enhancer, are compounds derived from the nucleotide metabolism. Biosynthesis of riboflavin requires two precursors, namely the pentose phosphate pathway intermediate ribulose 5-phosphate and the nucleotide guanosine 5'-triphosphate (GTP), which is derived from the purine metabolism (Figure 7). Inosinic acid, which is also designated inosine 5'-monophosphate (IMP), is a pathway intermediate in the purine metabolism leading to biosynthesis of GTP. Thus, it is not surprising that similar metabolic engineering strategies were followed for establishing production of riboflavin and inosinic acid.

Microbially produced riboflavin is typically obtained from the filamentous fungus *Ashbya gossypii* or from the yeast *Candida famata* as both are natural riboflavin overproducers (Stahmann et al., 2000). *A. gossypii* was extensively engineered toward increased riboflavin titers in the recent years. To this end, rational approaches were followed to increase the carbon fluxes into the purine and riboflavin biosynthetic pathways. It turned out that reduced expression of the adenylosuccinate synthase gene *ADE12* led to an increased flux from IMP into the guanosine monophosphate (GMP) branch and reduced the competing flux into the adenosine monophosphate (AMP) branch. The



overexpression of five genes coding for enzymes of the riboflavin synthesis pathway in a strain with reduced *ADE12* expression allowed for the production of 0.52 g/L riboflavin (Ledesma-Amaro et al., 2015). Very recently, the riboflavin production capabilities were further improved based on a  $^{13}\text{C}$  metabolic network analysis, which improved the final titer by 45% (the actual titer is not mentioned) (Schwechheimer et al., 2018). Metabolic engineering work in *E. coli* focused on overexpression of the native riboflavin biosynthesis genes *ribABDEC* along with additional modifications leading to reduced by-product formation and an increased flux into the pentose phosphate pathway (Lin et al., 2014). The best strain produced 2.7 g/L riboflavin with a yield of 0.14 g/g glucose. In engineered *B. subtilis* strains, riboflavin titers of 4.9 g/L were obtained (Wang et al., 2014).

Already in the 1960s, it was found that an adenine-auxotrophic *Micrococcus glutamicus* strain accumulated inosinic

acid to a concentration of 0.75 g/L ( $\text{IMP Na}_2 \times 7.5 \text{ H}_2\text{O}$ ) (Nakayama et al., 1964). Production was also achieved in *Corynebacterium ammoniagenes* strains obtained after random mutagenesis experiments, which were capable of accumulating 7.5 g/L inosinic acid (Tomita et al., 1991). Production of inosine, which can be obtained from inosinic acid by one dephosphorylation step, was demonstrated in *B. subtilis* (Asahara et al., 2010). The bacterium was engineered by initially deleting the genes coding for enzymes involved in the conversion of inosinic acid to GMP and AMP. Subsequently, expression of purine biosynthetic genes organized in the 12-gene *pur* cluster was deregulated by deletion of the gene encoding the regulator PurR and by removing a riboswitch. The resulting mutant strain was shown to produce 6 g/L inosine from 30 g/L glucose (Asahara et al., 2010). Enzymes suitable for the required conversion of inosine into inosinic acid were characterized *in vitro* (Liu et al., 2012) and were more recently also applied in a whole-cell



biotransformation (Yuan et al., 2016). As an alternative strategy, inosinic acid can also be produced by deamination of AMP (Li et al., 2017).

## Oligopeptides and Proteins

The E numbers catalog also includes oligopeptides and proteins, which serve as preservatives, sweeteners or antibiotics. Nisin (E234), an antibiotic oligopeptide consisting of 34 amino acids, is naturally produced by *L. lactis* and shows activity against Gram-positive bacteria (Severina et al., 1998). Nisin is encoded by the gene *nisA* and thus produced by ribosomes and not by nonribosomal peptide synthetases as many other peptide antibiotics (Kaletta and Entian, 1989). Strains of the natural producer *L. lactis* produced nisin by fermentation of milk or whey. It turned out that utilization non-filtrated milk whey allows for much higher nisin titers compared to filtrated milk whey. With different *L. lactis* strains nisin titers of 11.1 g/L were obtained (de Arauz et al., 2008). Metabolic engineering efforts toward increased production of nisin in *L. lactis* have been reviewed very recently (Özel et al., 2018).

Thaumatococin (E957) is a mixture of six proteins (thaumatin I, II, III, a, b, and c, all consisting of 207 amino acids). These sweet-tasting proteins are produced in berries of the plant *Thaumatococcus daniellii* (Wel and Loeve, 1972), which grows in western and central Africa (Adansi and Holloway, 1975). Already in 1982, one of the thaumatin genes was functionally expressed in *E. coli* (Edens et al., 1982). Later, thaumatin II was produced in *Aspergillus awamori* strains as well as in *B. subtilis* by expression of a codon-optimized thaumatin II gene and subsequent protein secretion (Illingworth et al., 1988; Moralejo et al., 1999). Thaumatin production in *A. awamori* was improved by deleting a gene encoding the protease aspergillopepsin B and by simultaneous overexpression of *bipA* encoding an endoplasmic reticulum chaperone. With this strategy, a titer of 13 mg/L properly folded thaumatin II was obtained (Moralejo et al., 2002; Lombraña et al., 2004).

Due to its antimicrobial activity lysozyme (E1105) is used as a preservative in foodstuff. Lysozyme cleaves 1,4- $\beta$ -bonds in peptidoglycan, which disturbs the integrity of the cell wall and leads to lysis of bacterial cells (Mir, 1977). Currently, commercially available lysozyme is obtained from egg white, which has several drawbacks, e.g., it requires laborious purification and can cause immunological problems in humans. As an alternative to extraction of lysozyme from egg white, recombinant *A. niger* was shown to be capable of producing this enzyme with a titer of 0.21 g/L (Gheshlaghi et al., 2005). In the recent years, there was increased interest in establishing microbial production of human lysozyme with higher activity compared to hen egg lysozyme, e.g., using *Pichia pastoris*, *E. coli*, *S. cerevisiae*, and *Kluyveromyces lactis* (Ercan and Demirci, 2016). Titers in the range of 0.03–0.13 g/L human lysozyme were obtained by expressing the human gene in microbial host organisms.

## Macrolide Antimicrobials

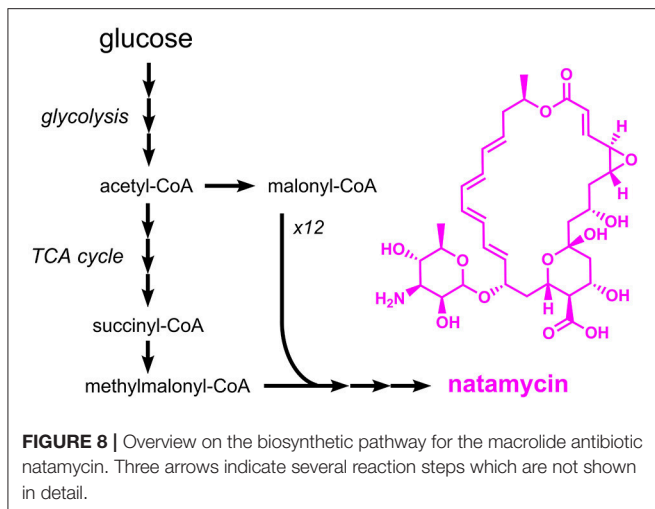
Natamycin (E235), a macrolide antibiotic from *Streptomyces natalensis*, has a similar mechanism of action as nisin (formation

of pores in cell membranes), but acts exclusively against fungi, e.g. *Candida*, *Aspergillus* and *Penicillium* species (Pedersen, 1992). Natamycin is approved as a preservative for the surface treatment of hard or semi-hard cheese. It is also used for dried and salted sausages. Although nisin and natamycin share a similar mechanism of action, both compounds are structurally and metabolically unrelated. Production of natamycin is initiated by a polyketide synthase which uses methylmalonyl-CoA and 12 molecules of malonyl-CoA as substrates (Liu et al., 2015) (Figure 8). The resulting compound, referred to as natamycinolide, is further modified by carboxylation, glycosylation, and epoxidation, which give rise to natamycin. The biosynthesis pathway for natamycin is highly complex and (amongst others) involves the large polyketide synthases PimS1 and PimS2 with a length of 6,797 and 9,507 amino acids, respectively. Thus, it is not surprising that production of natamycin focused on natural producers such as *S. natalensis* and closely related species. The natamycin production capabilities of wild-type *S. natalensis* were optimized by testing different sources of carbon, nitrogen and phosphate. With glucose, potassium dihydrogen phosphate and a mixture of beef extract and yeast extract production of 1.5 g/L natamycin was observed (Farid et al., 2000). In a related study, it was tested whether the supplementation of short-chain acids or alcohols has a positive impact on natamycin production. As a result, it turned out that 1-propanol supplementation increased the natamycin titer to 10.4 g/L, which was 17% higher than the control strain without supplementation (Li et al., 2014a). The same effect was also observed for the supplementation of acetic acid and propionic acid (Elsayed et al., 2013). In this study a final titer of 4.0 g/L natamycin was achieved. Natamycin production in the natural producer *Streptomyces gilvosporeus* was improved by coupling the expression of the natamycin biosynthetic gene cluster to the expression of a kanamycin resistance gene (Wang et al., 2016). After seven iterative rounds of mutagenesis and selection for increased kanamycin resistance a strain producing 14.4 g/L natamycin was obtained.

## DISCUSSION

Forty-two of the 316 compounds currently approved as food additives by the EU can in principle be produced in microorganisms until today (Table 1). It is impressive that a broad range of different compounds ranging from small organic acids to more complex secondary metabolites or polymers such as oligopeptides can now be accessed by tailor-made microbial cell factories (Table 2). Engineering efforts toward metabolite overproduction follow some basic strategies or principles, which generally apply for all tested microorganisms. These include increasing the activity of enzymes catalyzing rate-limiting reactions, the abolishment of natural regulatory circuits, e.g., transcriptional control of gene expression or allosteric feedback inhibition mechanisms, and the elimination of complex networks of competing pathways for preventing side product formation. Strain modification also targets increased carbon source uptake or product export or an increased resistance of





the host against the product or a pathway intermediate. In addition to increasing product titers there is often also room for reduction of production costs. This can e.g., be achieved by stable integration of heterologous genes into the host genome and expression from constitutive promoters, which allows for avoiding the use of antibiotics (for plasmid maintenance) and inducer compounds. In several cases, changing the expression rate of endogenous genes can be superior to gene deletion. This is especially true when deletions lead to auxotrophic strains, which can be circumvented by downregulating the gene instead of deleting it.

Interestingly, the range of host organisms exploited for production of food additives is not restricted to commonly used organisms such as *E. coli* and *S. cerevisiae*, but comprises a broad spectrum of bacteria, fungi, and microalgae (Table 2). Although it is reasonable to use established platform organisms, for which a wealth of metabolic engineering tools is available, it is also important to sample the natural diversity of microorganisms (Pei and Schmidt, 2018). This is in particular true for those organisms already naturally overproducing compounds of interest. Here, the development of tools for strain modification not only serves for increasing the overall product titers. It also contributes to extend our knowledge of the microbial physiology and its diversity and might enable identification of novel compounds or pathway intermediates. This information might also enable the use of alternative (or synthetic) pathways for production.

Currently, production approaches for approved food additives are at three different stages reflecting economical viability of large-scale production, which are designated stage I, II, and III here. The titers required for an economically viable production (corresponds to stage III) strongly depend on the complexity and value of the product, thus only a rough classification for each stage can be given here. For several products microbial production is already economically viable and industrial processes are well-established, e.g., for L-glutamate, riboflavin, L-ascorbic acid, succinic acid, and lactic acid, which can be classified as stage III. For these compounds product titers of 100 g/L or more are typically obtained and also methods

and processes for product purification are established. Stage II comprises products, for which titers in the range of 10–100 g/L are obtained. For such products titers obtained with microorganisms are close to economical viability and it can be expected that industrial production is in reach. Examples for products at stage II are adipic acid and one-ring aromatics such as 4-hydroxybenzoic acid. As one example, the company Verdezyne opened a pilot plant for bio-based adipic acid production with yeast in 2011 (Tetzlaff, 2011). Current challenges during microbial overproduction of stage III and II compounds are metabolic imbalances in the producer strain (e.g., with regard to the availability of reducing equivalents or ATP), or a depletion of metabolites required for sufficient biomass formation, but also product toxicity and insufficient product export. These challenges can be addressed by additional strain engineering and by choosing suitable process conditions (e.g., using two stage cultivation strategies with a production phase decoupled from biomass formation).

Products with titers in the mg/L range belong for the most part to stage I. In particular for more complex plant-derived polyphenols and terpenoids titers obtained in engineered microorganism are often still too low for establishing production at larger scale and today most of these compounds are still obtained by extraction from plants containing the desired compounds in larger amounts. Major challenges during production of plant natural products with microorganisms are often directly associated to their bioactivity as many of them have anti-microbial activities or tend to react with oxygen or radicals (anti-oxidants). For these products the optimization of cultivation conditions should focus on preventing oxidation and on reducing toxicity for the production host.

As only naturally-occurring metabolic pathways were exploited for food additive production in engineered microorganisms all compounds discussed in the main section of this review article are natural compounds. This is somehow self-evident when taking into consideration that synthetic compounds can typically not be produced using natural metabolic pathways. On the contrary, this does not mean that only pathways evolved by nature must be followed for production. One recent example is the plant-derived stilbene resveratrol, which is naturally produced from the phenylpropanoid *p*-coumaric acid. For circumventing a bottleneck reaction at the stage of aromatic amino acids the functional reversal of a bacterial catabolic pathway was exploited as a novel route toward resveratrol production (Kallscheuer et al., 2016). In another study, the relaxed substrate specificity of enzymes was exploited for producing small organic compounds, which cannot be obtained by pathways evolved by nature (Cheong et al., 2016).

In the recent years there was an increasing interest in replacing synthetic food additives by their natural counterparts. This is due to that fact that many synthetic food additives were shown to have negative effects on human health. Scientists have been pointing out for years that synthetic dyes may be involved in the development of attention deficit hyperactivity syndrome (ADHD) (Stevens et al., 2013). Since July 2010, the EU has made the warning label “May affect the activity and

**TABLE 2** | Production of food additives approved by the EU in engineered microorganisms.

E number	Compound	Production host	Carbon source or precursor	Product titer (g/L)	Yield (g/g)	References
<b>CARBOXYLIC ACIDS AND SHORT-CHAIN ALCOHOLS</b>						
E270	Lactic acid	<i>Bacillus</i> sp.	Glucose	225	0.99	Meng et al., 2012
E296	Malic acid	<i>Aspergillus oryzae</i>	Glucose	154	1.02	Brown et al., 2013
E297	Fumaric acid	<i>Rhizopus oryzae</i>	Glucose	85	0.85	Cao et al., 1996
E330	Citric acid	<i>Aspergillus niger</i>	Glucose	157	0.98	Yin et al., 2017
E334	Tartaric acid	<i>Gluconobacter oxydans</i>	Sorbitol	2.1	0.11	Chandrashekar et al., 1999
E355	Adipic acid	<i>Escherichia coli</i>	Glycerol	2.5	0.12	Cheong et al., 2016
E363	Succinic acid	<i>Escherichia coli</i>	Glycerol	96	0.96	Grabar et al., 2014
E422	Glycerol	<i>Candida glycerinogenes</i>	Glucose	130	0.63	Zhuge and Fang, 1995
E1520	1,2-Propanediol	<i>Clostridium thermosaccharolyticum</i>	Glucose	7.9	0.27	Cameron and Cooney, 1986
<b>AMINO ACIDS</b>						
E620	L-glutamic acid	<i>Corynebacterium glutamicum</i>	Glucose	100	0.6	Ault, 2004
E920	L-cysteine	<i>Escherichia coli</i>	Glucose	1.7	0.06	Takagi et al., 1999
<b>TERPENOIDS</b>						
E160a	$\beta$ -Carotene	<i>Escherichia coli</i>	Glycerol	0.47	0.02	Yoon et al., 2009
E160d	Lycopene	<i>Escherichia coli</i>	Glycerol	1.4	0.04	Zhu et al., 2015
E161b	Lutein	<i>Muriellopsis</i> sp.	CO <sub>2</sub>	0.035	–	Del Campo et al., 2000
E161g	Canthaxanthin	<i>Dietzia natronolimnaea</i>	Glucose	0.008	3.2*10 <sup>-4</sup>	Gharibzahedi et al., 2012
E307	$\alpha$ -Tocopherol	<i>Euglena gracilis</i>	Tyrosine, homogenisate	0.14	–	Tani and Tsumura, 1989
<b>ONE-RING AROMATICS AND POLYPHENOLS</b>						
E100	Curcumin	<i>Escherichia coli</i>	Ferulic acid (in rice bran pitch)	0.06	–	Katsuyama et al., 2008
E162	Betanin	<i>Saccharomyces cerevisiae</i>	Dextrose	0.017	8.5*10 <sup>-4</sup>	Grewal et al., 2018
E163	Callistephin (an anthocyanin)	<i>Escherichia coli</i>	Glucose	0.010	5.0*10 <sup>-4</sup>	Jones et al., 2017
E214	4-Hydroxybenzoic acid	<i>Corynebacterium glutamicum</i>	Glucose	36.6	0.31	Kitade et al., 2018
E310	Gallic acid	<i>Escherichia coli</i>	Glucose	20	0.12	Kambourakis et al., 2000
E1519	Benzyl alcohol	<i>Escherichia coli</i>	Glucose	0.14	0.008	Pugh et al., 2015
<b>SUGAR-DERIVED COMPOUNDS</b>						
E300	2-Keto-L-gulonic acid (ascorbic acid precursor)	<i>Gluconobacter oxydans</i>	Sorbitol	130	0.87	Saito et al., 1997
E420	Sorbitol	<i>Zymomonas mobilis</i>	Glucose + fructose	233	1.0	Bringer-Meyer and Sahn, 1991
E421	Mannitol	<i>Corynebacterium glutamicum</i>	Fructose	87	0.93	Bäumchen and Bringer-Meyer, 2007
E574	Gluconic acid	<i>Aspergillus niger</i>	Glucose	311	1.05	Lu et al., 2015
E967	Xylitol	<i>Kluyveromyces marxianus</i>	Xylose	312	0.99	Zhang et al., 2015a
E968	Erythritol	<i>Pseudozyma tsukubaensis</i>	Glucose	243	0.61	Jeya et al., 2009
<b>NUCLEOTIDE-DERIVED COMPOUNDS</b>						
E101	Riboflavin	<i>Bacillus subtilis</i>	Glucose	4.9	0.05	Wang et al., 2014
E630	Inosinic acid	<i>Corynebacterium ammoniagenes</i>	Glucose	7.5	0.15	Tomita et al., 1991
<b>OLIGOPEPTIDES AND PROTEINS</b>						
E234	Nisin	<i>Lactococcus lactis</i>	Glucose	11.1	–	de Arauz et al., 2008
E957	Thaumatococin	<i>Aspergillus awamori</i>	Glucose	0.013	–	Moralejo et al., 2002
E1105	Lysozyme	<i>Aspergillus niger</i>	Glucose	0.21	–	Gheshlaghi et al., 2005
<b>ANTIBIOTICS</b>						
E235	Natamycin	<i>Streptomyces natalensis</i>	Complex ingredients	14.4	–	Wang et al., 2016

attention of children” mandatory for all manufacturers using the controversial substances. The new regulation applies to the dyes tartrazine (E102), quinoline yellow (E104), yellow-orange S (E110), azorubine (E122), cochineal red (E124), and allured (E129). Also negative effects on health of artificial sweeteners are currently discussed. Cyclamic acid is banned in the US and UK due its potential links to cancer, but it is still approved as food additive in the EU as E952. Also the role of artificial sweeteners in the prevention of obesity is controversially discussed (Suez et al., 2014).

In contrast, natural products approved by the EU are generally recognized as safe, well-accepted by customers and some even show health-promoting effects (Vauzour et al., 2010). It can be expected that future efforts for increasing microbial production of such natural compounds will profit from novel methods such as CRISPR/Cas9 and from combination of rational strain engineering, adaptive laboratory evolution and high-throughput screening approaches (Schallmeyer et al., 2014; Eggeling et al., 2015;

Shalem et al., 2015). One promising example are transcription factor-based biosensors for screening of strain libraries, e.g., for production of L-lysine, succinic acid, adipic acid, and also for plant polyphenols such as naringenin or quercetin (Eggeling et al., 2015). It is highly likely that such novel synthetic biology approaches will render microbial production especially of many plant natural compounds economically viable within the next 10–15 years.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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# Advantages of Using Blend Cultures of Native *L. plantarum* and *O. oeni* Strains to Induce Malolactic Fermentation of Patagonian Malbec Wine

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The malolactic fermentation (MLF) of Patagonian Malbec wine inoculated with blend cultures of selected native strains of *Lactobacillus plantarum* and *Oenococcus oeni* was monitored during 14 days, analyzing the strains ability to modify the content of some organic acids and to change the volatile compounds profile. The performance of the LAB strains was tested as single and blends cultures of both species. An implantation control by RAPD PCR was also carried out to differentiate among indigenous and inoculated strains. The *L. plantarum* strains UNQLp11 and UNQLp155 and the *O. oeni* strain UNQOe73.2 were able to remain viable during the monitoring time of MLF, whereas the *O. oeni* strain UNQOe31b showed a decrease of five log CFU at day 14. The four strains assayed showed a similar behavior in wine whether they were inoculated individually or as blend cultures. All strains were able to consume L-malic acid, particularly the *L. plantarum* strains, which showed the highest consumption values at day 14, both as single or blend cultures. The changes in the volatile compounds profile of Malbec wine samples, before and after MLF, were determined by HS-SPME and GC-MS technique. Wines inoculated with blend cultures containing strain UNQLp155 showed a decrease in the total alcohols content and an increase in the total esters content. On the other hand, wines inoculated with single cultures of strains UNQLp155, UNQOe31b or UNQOe73.2 showed no significant decrease in the total alcohols concentration but a significant increase in the total esters content. When strain UNQLp11 was inoculated as single or as blend culture with strain UNQOe31b, wines exhibited an increase in the total alcohols content, and a decrease in the total esters content. The content of diethyl succinate



showed the greatest increase at final of MLF, and a particular synergistic effect in its synthesis was observed with a blend culture of strains UNQLp155 and UNQOe73.2. These results suggest that the use of blend cultures formulated with strains belonging to *L. plantarum* and *O. oeni* species could offer an interesting advantage to induce MLF in Malbec wines, contributing to diversify their aromatic profiles.

**Keywords:** *L. plantarum*, *O. oeni*, Patagonian Malbec wine, flavor, L-malic acid

## INTRODUCTION

Lactic acid bacteria perform MLF, an important step in the red grapes winemaking. Wine deacidification is the main consequence of the conversion of L-malic to L-lactic acid, resulting in a decrease in titrable acidity of wine and a small increase of pH. MLF also leads to enhanced microbial stability and is usually believed to improve the complexity of the wine aroma (Pozo-Bayón et al., 2005; Renouf et al., 2005). The biosynthesis of aroma compounds during MLF includes the activity of a broad range of enzymes present in LAB, such as glycosidases, esterases, phenolic acid decarboxylases and citrate lyases, whose activities may affect wine aroma and complexity. Different studies have focused on the biosynthesis of aroma compounds during MLF and the concomitant organoleptic consequences (Ugliano et al., 2003; Costantini et al., 2009). On the other hand, the influence of LAB strains on wine aroma composition and complexity is not yet well-known. Different authors have shown that aroma/flavor wine attributes can vary according to LAB strains used in MFL induction (Gámbaro et al., 2001; Boido et al., 2009; Iorizzo et al., 2016; Cappello et al., 2017).

Although MLF often occurs spontaneously, by action of native LAB from grapes and cellar, it implies risks such as a considerable increase in the volatile acidity, consumption of residual sugars, and formation of undesirable metabolites such as biogenic amines (Mira de Orduña et al., 2000; Liu, 2002; Marcobal et al., 2006). In order to avoid losses in production, the use of commercial MLF starter cultures is normally recommended. However, the available commercial starters may have been formulated with LAB strains isolated from regions different to those in which they are going to be used and thus, adding a variability factor in wine production. The use of indigenous starter cultures best adapted to a specific wine-producing area is therefore recommended to maintain the wine regional characteristics (du Toit et al., 2011; Garofalo et al., 2015; Berbegal et al., 2016).

Malolactic fermentation of Patagonian red wines occurs spontaneously and the prevalence of strains belonging to *Oenococcus oeni* and *Lactobacillus plantarum* species during this winemaking step suggests that these strains are involved in leading the spontaneous MLF of Pinot noir and Merlot wines (Valdés La Hens et al., 2015). In previous works, a great number of strains of both species have been isolated and characterized regarding their oenological and technological

properties (Bravo-Ferrada et al., 2013, 2014; Brizuela et al., 2017). A recent study regarding the behavior of selected native strains inoculated in sterile Pinot noir wine, and their ability to change the volatile compounds profile of wine (Brizuela et al., 2018), showed a decrease in the alcohols content and an increase in the volatile esters content, particularly when *O. oeni* strains were inoculated. Meanwhile, the *L. plantarum* strains were more efficient to consume the L-malic acid. With this background, the goal of this work was to study the effect of single or blend cultures of native strains of *O. oeni* y *L. plantarum* species to induce MLF of a Patagonian Malbec wine, investigating changes in the volatile compounds and in some organic acids content.

## MATERIALS AND METHODS

### Wine Sample

A Patagonian Malbec wine vintage 2016, at final stage of AF (12.4% v/v ethanol, pH 3.6, <2.00 g/L residual sugars, 2.0 g/L-malic acid, 96 mg/L total SO<sub>2</sub>, total acidity 3.98 g/L) was employed. In this wine, the AF was carried out with the native Patagonian F8 *Saccharomyces cerevisiae* strain (Simes et al., 2016).

### *O. oeni* and *L. plantarum* Strains

The selected Patagonian LAB strains used were: *L. plantarum* UNQLp11, and UNQLp155, and *O. oeni* UNQOe31b, and UNQOe73.2. These strains were isolated from Patagonian Pinot noir wines (vintages 2008 and 2014) and selected according to their oenological properties (Bravo-Ferrada et al., 2013, 2016; Brizuela et al., 2017).

### Cell Cultures and Acclimation

*Lactobacillus plantarum* strains were grown in MRS (Biokar Diagnostic, Beauvais, France) (De Man et al., 1960), and *O. oeni* strains were grown in MLO medium (Maicas et al., 1999). Bacterial cell cultures in the early stationary phase ( $\sim 10^9$  CFU/mL) were collected by centrifugation at 5,000 RPM for 10 min and suspended in the same volume of an acclimation medium (50 g/L MRS, 40 g/L D(-) fructose, 20 g/L D (-) glucose, 4 g/L L-malate, 1 g/L Tween 80, and 0.1 mg/L pyridoxine, adjusted to pH 4.6) supplemented with 6% v/v ethanol (Bravo-Ferrada et al., 2014). Cultures were incubated during 48 h at 21°C according to Brizuela et al. (2017).

### Vinification Assays at Laboratory Scale

Acclimated cells were harvested by centrifugation and inoculated ( $\sim 5 \times 10^7$  CFU/mL) in 80 mL of wine to induce MLF. LAB strains were inoculated as single cultures (UNQLp11,

**Abbreviations:** AF, Alcoholic fermentation; GC-MS, gas chromatography-mass spectrometry; HS-SPME, Headspace solid phase microextraction; LAB, Lactic Acid Bacteria; MLF, Malolactic Fermentation.



UNQLp155, UNQOe31b, and UNQOe73.2) or blend cultures (UNQLp11/UNQOe31b, UNQLp11/UNQOe73.2, UNQLp155/UNQOe31b, and UNQLp155/UNQOe73.2), and wine samples were incubated at 21°C during 14 days. For blend cultures only a half concentration of each strain was inoculated, in order to obtain a final concentration of  $\sim 5 \times 10^7$  CFU/mL. Control sample was not-inoculated Malbec wine, incubated in the same conditions that inoculated wine samples. Values of cell survival were determined by plating on MRS (*Lactobacillus*) or MLO (*O. oeni*) agar plates added with 100 mg/L of cycloheximide (Sigma, United States) and 20 mg/mL of nystatin (Sigma-Aldrich, Argentina).

## Implantation Strain Ability

Implantation strain ability in non-sterile Malbec wine samples was performed by Random Amplified Polymorphic DNA (RAPD) method. From each sample, before and after MLF, ten colonies were randomly chosen from MLO and/or MRS plates and inoculated in MLO or MRS broth, respectively, to obtain DNA from each culture. All colonies were characterized as LAB by Gram-positive staining, negative catalase, and morphology was observed. DNA extraction was performed according to Bravo-Ferrada et al. (2011). DNA samples were quantified using a Nanodrop spectrophotometer (Thermo Scientific, 1000) and visualized on a 1.0% (w/v) agarose gel. *Oenococcus* and *Lactobacillus* isolates were typed by RAPD-PCR analysis using primer M13 (Stenlid et al., 1994). Amplification reactions were performed according to Delfederico et al. (2006), and PCR products were separated on a 2.0% (w/v) agarose electrophoresis gel using a 100 bp ladder PB-L (Productos Bio-Lógicos, UNQ). The evaluation of implantation ability was performed by comparing the RAPD profiles of each colony with profiles of the inoculated strains.

## Organic Acids

Concentrations of L- malic, tartaric, citric, and L-lactic acids were measured at day 0 and 14th using the Enology BioSystems kits, according to manufacturer instructions (L-malic acid, Tartaric acid, Citric Acid, and Lactic acid, BioSystems SA, Barcelona, Spain).

## Headspace Solid Phase Microextraction (HS-SPME)

Headspace solid phase microextraction was employed for volatile compounds sampling following the protocols previously described (Rodríguez-Bencomo et al., 2011), with modifications. Briefly, 8 mL of wine or hydroalcoholic solution containing the aroma compounds were placed in a 20 mL headspace vial with 40  $\mu$ L of the three internal standards (3-octanol, methylnonanoate, and 3,4-dimethylphenol), and sealed with a TFE/silicone septum (Supelco, Bellefonte, PA, United States). Samples were left in a water bath at 40°C for 5 min before the extraction. The extraction was performed with the exposure of a Stable Flex 85  $\mu$ m carboxen-polydimethylsiloxane, CAR-DVB-PDMS fiber (Supelco) to the headspace of the sample for 10 min at 40°C and under constant stirring (500 rpm). After

the extraction, the fiber was removed from the sample vial and desorbed in the GC injector port in splitless mode for 80 min. Six levels of concentration of each aroma compound (2, 10, 100, 500, 1000, 5000  $\mu$ g/L), covering the concentration ranges expected in wines, were tested in duplicate. Prior to use, the fiber was conditioned following the supplier's recommendation.

## Gas Chromatography–Mass Spectrometry Analysis

An Agilent 7890A GC system (Agilent, Palo Alto, CA, United States), with a split/splitless injector and interfaced with an Agilent 5975N mass spectrometer was used for volatile compounds analysis. The injector was set at 250°C. The Agilent MSD Chem Station Software (D.01.02 16 version) was used to control the system. Volatile compounds were separated on a DB-Wax polar capillary column (60 m  $\times$  0.25 mm i.d.  $\times$  0.50  $\mu$ m film thickness) from Agilent (J&W Scientific, Folsom, United States). Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature was programmed as follows: an initial temperature of 40°C which was maintained during 5 min, and then increased to 240°C (4°C/min) which was kept for 15 min. For the MS system, the temperatures of the transfer line, quadrupole and ionization source were 270, 150, and 230°C respectively; electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10A. The acquisitions were performed in Scan (from 35 to 450 amu) and SIM modes. Peak identification was carried out by analogy of mass spectra with those of the mass library (Wiley 6.0 and NIST 2.0), and with those from reference compounds analyzed in the same conditions that wine samples. Quantitative data were obtained by calculating the relative peak area (or TIC signal) in relation to that of the three internal standards used (3-octanol, methylnonanoate and 3,4-dimethylphenol), depending on the volatile compound. Calibration curves of each compound were performed using a hydroalcoholic solution (pH 3.6, 14% v/v ethanol) spiked with the commercial pure reference compounds at six levels of concentration (2, 10, 100, 500, 1000, 5000  $\mu$ g/L) covering the concentration ranges expected in wine and tested in duplicate.

The aroma standard solutions for the calibration curve were prepared in HPLC grade absolute ethanol supplied by Merck. The 51 compounds used were: butyl acetate (123-86-4), ethyl hexanoate (123-66-0), ethyl decanoate (110-38-3) and vanillin (121-33-5) from Merck (Darmstadt, Germany); isobutyl acetate (110-19-0), ethyl butanoate (105-54-4), isopentyl acetate (123-92-2), hexyl acetate (142-92-7), 1-hexanol (111-27-3), *cis*-3-hexen-1-ol (928-96-1), ethyl octanoate (106-32-1), furfural (98-01-1), linalool (78-70-6),  $\gamma$ -butyrolactone (96-48-0), diethyl succinate (123-25-1),  $\alpha$ -terpineol (98-55-5),  $\beta$ -damascenone (23726-91-2), 2-phenylethyl acetate (103-45-7), geraniol (106-24-1), guaiacol (90-05-01), whiskey lactone (39212-23-2),  $\alpha$ -ionone (79-77-6) and eugenol (97-53-0) from Sigma-Aldrich; hexanoic acid (142-62-1), and decanoic acid (334-48-5) from Scharlau (Barcelona, Spain) and 4-ethyl guaiacol (2785-89-9) from Lancaster (Eastgate, White Lund, Morecambe, England);  $\alpha$ -pinene,  $\beta$ -pinene, limonene, terpinen-4-ol,  $\beta$ -citronellol, nerol, 5-methylfurfural, furfuryl alcohol, benzyl alcohol,  $\beta$ -phenylethyl

alcohol, decanoic acid, 2,3-butanodione, ethyl propanoate, 1-butanol, ethyl 2-methylbutyrate, *trans*-3-hexen-1-ol,  $\beta$ -ionone,  $\gamma$ -nonalactone, ethyl cinnamate, 4-ethylphenol, 2-methoxy-4-vinylphenol, 2,6-dimethylphenol, methyl vanillate, ethyl vanillate, acetovanillone, ethyl dodecanoate from Sigma-Aldrich. These compounds were selected for their important role for wine aroma, being representative of the wine volatile profile. The aroma standards were purer than 98%. All the solutions were stored at 4°C.

## Reproducibility of the Results

Three vinification assays were carried out using single or blend cultures and all the experiments, for each sample, were done, at least, in duplicate. The statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, 2007). Data are presented as mean  $\pm$  SD and compared by one-way ANOVA followed by a Tukey or Dunnett post-test for multiple comparisons, and if  $p < 0.05$  the difference was considered statistically significant.

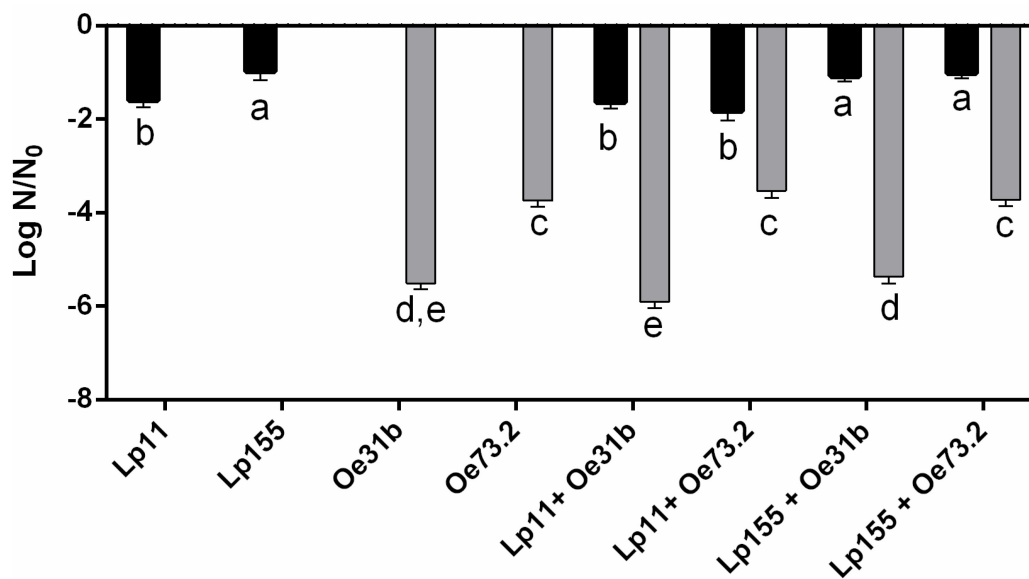
## RESULTS

Based on previous studies of oenological and technological behavior of native *L. plantarum* and *O. oeni* strains from Patagonian Pinot noir wines, by inoculation in wine-like media and in sterile wine, four strains were selected: two *L. plantarum* (UNQLp11, UNQLp155), and two *O. oeni* (UNQOe31b and UNQOe73.2). These strains were inoculated as single or blend

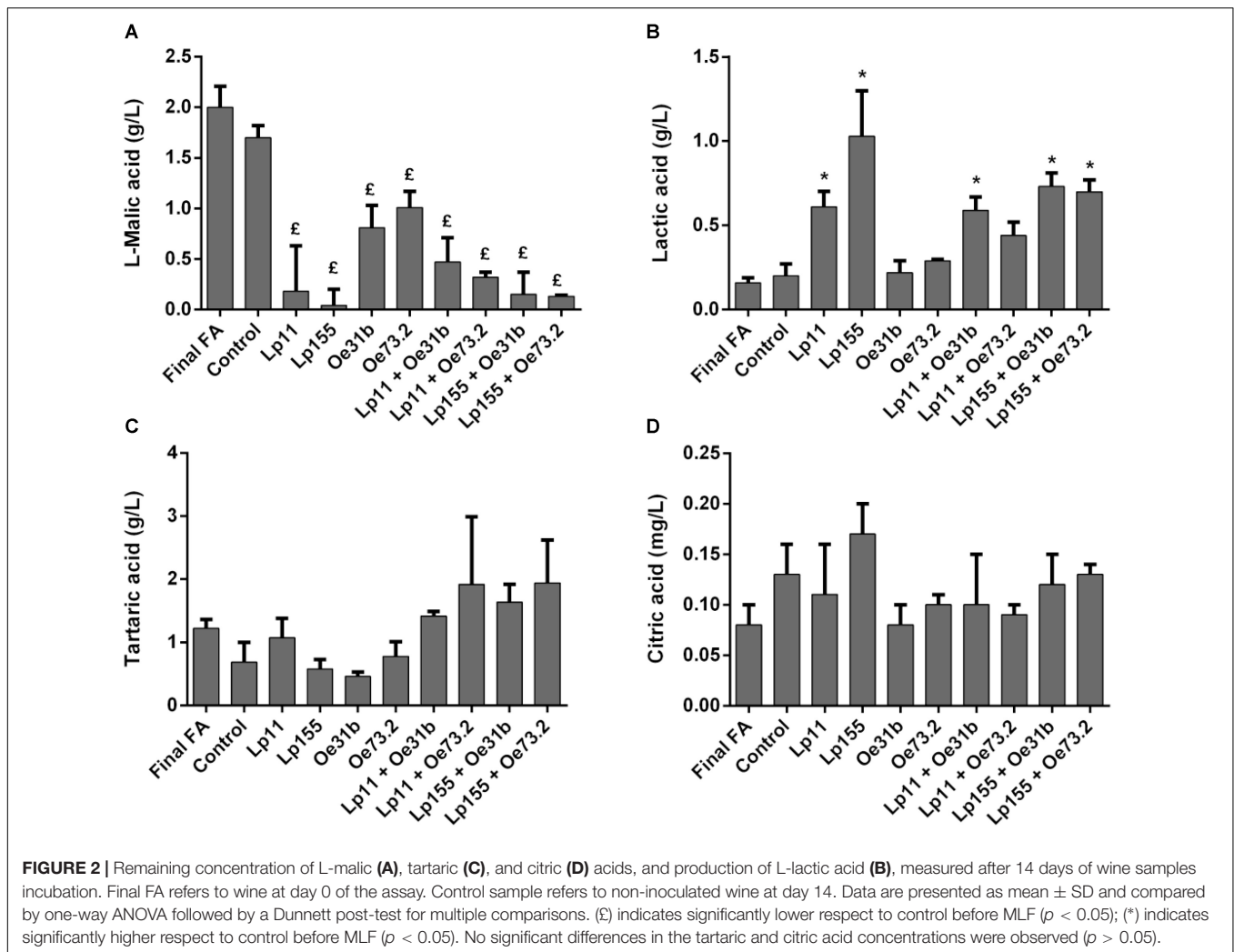
cultures in samples of a non-sterile Patagonian Malbec wine, at final stage of AF, with the aim to analyze their ability to perform the MLF in presence of wine natural microbiota. The viable cell counts in Malbec wine samples at final stage of AF were  $\sim 1 \times 10^5$  CFU/mL of *Lactobacillus* and  $\sim 1 \times 10^4$  CFU/mL of *O. oeni*.

**Figure 1** shows the loss of cell viability of single and blend cultures ( $\sim 5 \times 10^7$  CFU/mL) when were inoculated in Malbec wine and incubated during 14 days at 21°C. Values of viable cell counts of *O. oeni* strains showed a decrease of 4 to almost 6 log units, being the strain UNQOe31b less tolerant than strain UNQOe73.2. Instead, the viability of *L. plantarum* strains showed a lower decrease which did not exceed the 2 log units, being significantly lower for UNQLp155. For blend cultures the same behavior was observed as for single cultures. Blend cultures containing strain UNQOe31b exhibited the greatest loss of viability, whereas *L. plantarum* strains maintained a similar viability both as single and as blend cultures. In control wine sample (not inoculated), only *Lactobacillus* were detected after 14 days, indicating that *O. oeni* strains were not able to survive during this time. The implantation of cultures in non-sterile wine was controlled by RAPD PCR analysis with M13 primer. Percentages of implantation, at day 14, were: for single cultures, UNQLp11 25%, UNQLp155 43%, UNQOe31b 55%, and UNQOe73.2 23%, and for blend cultures, UNQOe31b (63%) + UNQLp11 (12.5%); UNQOe31b (60%) + UNQLp155 (25%); UNQOe73.2 (40%) + UNQLp11 (26%); UNQOe73.2 (37%) + UNQLp155 (35%) (data not shown).

**Figure 2** shows changes in the concentrations of L- malic, L-lactic, tartaric, and citric acids, before and after, vinification



**FIGURE 1** | Loss of viability expressed as Log N/N<sub>0</sub> of single cultures UNQLp11, UNQLp155, UNQOe31b, UNQOe73.2, and blend cultures UNQLp11/UNQOe31b, UNQLp11/UNQOe73.2, UNQLp155/UNQOe31b, and UNQLp155/UNQOe73.2 after 14 days of incubation in Malbec wine samples at 21°C. Gray bars indicate plate viable cell count number of strains belonging to *Oenococcus* species; black bars indicate viable cell counts of strains belonging to *Lactobacillus* species. Control wine showed no significant decrease in *Lactobacillus* count respect to day 0, but no *Oenococcus* species were detected after 14 days of MLF. Data are presented as mean  $\pm$  SD and compared by one-way ANOVA followed by a Tukey post-test for multiple comparisons. Different letters (a–e) denote statistically significant difference ( $p < 0.05$ ).



assays. The four single cultures were able to significantly consume L-malic acid, being UNQLp155 and blend cultures containing this strain those who consume the largest amount (Figure 2A). Control wine sample showed no significant consumption of L-malic acid, indicating the failure or delay of natural microbiota to conduct MLF.

Regarding L-lactic acid production (Figure 2B), only single cultures of *L. plantarum* strains (UNQLp11 and UNQLp155) and blend cultures (UNQLp11/UNQOe31b, UNQLp155/UNQOe31b, and UNQLp155/UNQOe73.2) were able to significantly increase its concentration during vinification assays. While the single culture UNQOe73.2 and blend culture UNQLp11/UNQOe73.2 were able to consume 50 and 85%, respectively, of L-malic acid, the increase in L-lactic acid concentration was not significant. In relation to changes in the content of tartaric (Figure 2C) and citric acids (Figure 2D), although they seem to show remarkable variations, there were no significant for any of cultures assayed ( $p > 0.05$ ).

Modifications in the volatile compounds profiles of Malbec wine samples were determined by HS-SPME gas chromatography technique. Changes in concentration of 5 alcohols, 9 esters,

1 terpene, and 3 other volatile compounds, before and after vinification assay, were monitored (Tables 1, 2 and Figure 3). Alcohols were the main volatile compounds in Malbec wine samples at final stage of AF. Although a decrease of 3-methyl-1-butanol, 1-butanol, and 1-hexanol was observed with the different cultures inoculated, the percentage of total alcohols only decreased when MLF was carried out by the single culture UNQLp155 and blend cultures UNQLp155/UNQOe31b, and UNQLp155/UNQOe73.2, noting also an increase in total esters content. Wine samples inoculated with the other three single cultures (UNQLp11, UNQOe31b, and UNQOe73.2) showed no significant changes in total alcohols content, but a significant increase in total esters content was observed. Wine sample inoculated with blend culture UNQLp11/UNQOe31b exhibited an increase in total alcohols content, and the one inoculated with single culture UNQLp11, showed a significant decrease in total esters content (Tables 1, 2 and Figure 3).

As can be seen in Table 1, all volatile compounds tested showed a decrease in its concentration values when vinification assay was carried out by single cultures, while esters content (Figure 3) showed an upward trend (except with UNQLp11

**TABLE 1** | Volatile compounds content (mg/L) in wine samples after (day 14) inoculation with cultures of the single strains.

Aromatic compounds (mg/L)	Control wine	<i>L. plantarum</i>		<i>O. oeni</i>	
		UNQLp11	UNQLp155	UNQOe31b	UNQOe73.2
<b>Alcohols</b>					
3-methyl-1-butanol	1.56 ± 0.18	0.71 ± 0.02 (£)	0.48 ± 0.02 (£)	0.53 ± 0.01 (£)	0.59 ± 0.03 (£)
1-butanol	4.28 ± 0.10	1.58 ± 0.07 (£)	nd (£)	1.07 ± 0.02 (£)	1.14 ± 0.02 (£)
1-hexanol	3.78 ± 0.06	1.09 ± 0.02 (£)	0.81 ± 0.01 (£)	0.95 ± 0.05 (£)	1.09 ± 0.17 (£)
Benzyl alcohol	Nd	0.12 ± 0.14	0.12 ± 0.01	nd	nd
β-phenyl ethyl alcohol	9.84 ± 0.09	8.93 ± 0.45	7.74 ± 0.19	10.01 ± 0	9.93 ± 1.35
<b>Esters</b>					
Isobutyl acetate	0.44 ± 0.05	0.11 ± 0.01 (£)	0.08 ± 0.01 (£)	0.09 ± 0.01 (£)	0.10 ± 0.01 (£)
Ethyl butyrate	1.33 ± 0.07	0.37 ± 0.03 (£)	0.29 ± 0.01 (£)	0.31 ± 0.03 (£)	0.35 ± 0.07 (£)
Isoamyl acetate	3.59 ± 0.15	0.99 ± 0.04 (£)	0.78 ± 0.01 (£)	1.01 ± 0.24 (£)	1.24 ± 0.48 (£)
Ethyl hexanoate	1.97 ± 0.11	0.80 ± 0.02	0.72 ± 0.05 (£)	0.87 ± 0.09	1.04 ± 0.32
Hexyl acetate	0.03 ± 0.08	0.01 ± 0	0.02 ± 0.01	0.017 ± 0	0.023 ± 0
Ethyl octanoate	1.45 ± 0.05	0.76 ± 0.04	0.07 ± 0.01 (£)	0.82 ± 0.03	0.46 ± 0.46 (£)
Ethyl decanoate	1.79 ± 0.17	0.74 ± 0.01 (£)	0.06 ± 0.03 (£)	0.748 ± 0.06 (£)	0.43 ± 0.49 (£)
Diethyl succinate	0.73 ± 0.15	0.49 ± 0.02	5.46 ± 0.31 (*)	5.95 ± 0.11 (*)	6.44 ± 0.20 (*)
Ethyl dodecanoate	0.25 ± 0.08	0.26 ± 0.01	0.04 ± 0.01 (£)	0.25 ± 0.01	0.24 ± 0
<b>Terpenes</b>					
β-citronellol	Nd	0.02 ± 0 (*)	nd	nd	0.02 ± 0.02 (*)
<b>Other compounds</b>					
2,3-butanodione	1.33 ± 0.17	nd (£)	nd (£)	nd (£)	nd (£)
γ-butyrolactone	9.99 ± 0.21	7.72 ± 0.81	7.31 ± 1.93	7.62 ± 1.44	8.20 ± 1.35
Furfural	0.09 ± 0.03	0.04 ± 0 (£)	0.03 ± 0 (£)	0.04 ± 0 (£)	0.04 ± 0 (£)

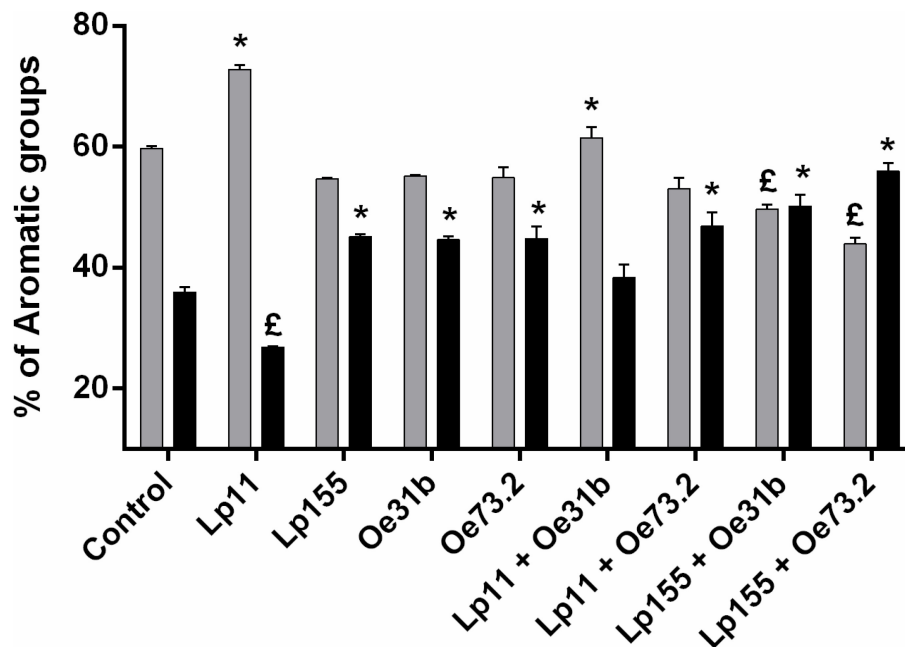
(\*): Significantly higher respect to control before MLF ( $P < 0.05$ ), (£): Significantly lower respect to control before MLF ( $P < 0.05$ ). nd: not detected. Control wine correspond to non-inoculated wine.

**TABLE 2** | Volatile compounds content (mg/L) in wine samples after (day 14) inoculation with cultures of blend cultures.

Aromatic compounds (mg/L)	Control wine	<i>Mixed cultures L. plantarum + O. oeni</i>			
		UNQLp11 + UNQOe 31b	UNQLp11 + UNQOe 73.2	UNQLp155 + UNQOe 31b	UNQLp155 + UNQOe 73.2
<b>Alcohols</b>					
3-methyl-1-butanol	1.56 ± 0.18	0.58 ± 0.11 (£)	0.65 ± 0.05 (£)	0.54 ± 0.07 (£)	0.77 ± 0.21 (£)
1-butanol	4.28 ± 0.06	1.22 ± 0.09 (£)	1.42 ± 0.15 (£)	1.145 ± 0.15 (£)	1.73 ± 0.40 (£)
1-hexanol	3.78 ± 0.10	1.12 ± 0.40 (£)	1.06 ± 0.07 (£)	0.98 ± 0.01 (£)	1.184 ± 0.15 (£)
Benzyl alcohol	Nd	0.22 ± 0.07	0.20 ± 0.02	0.13 ± 0.01	0.14 ± 0.04
β-phenyl ethyl alcohol	9.84 ± 0.09	8.88 ± 1.06	8.64 ± 1.61	9.11 ± 0.56	9.33 ± 0.27
<b>Esters</b>					
Isobutyl acetate	0.44 ± 0.05	0.13 ± 0 (£)	0.10 ± 0.01 (£)	0.08 ± 0 (£)	0.10 ± 0 (£)
Ethyl butyrate	1.33 ± 0.07	0.36 ± 0.15 (£)	0.32 ± 0.02 (£)	0.30 ± 0.02 (£)	0.37 ± 0.04 (£)
Isoamyl acetate	3.59 ± 0.15	0.90 ± 0.34 (£)	0.83 ± 0.08 (£)	0.73 ± 0.02 (£)	1.19 ± 0.39 (£)
Ethyl hexanoate	1.97 ± 0.11	0.98 ± 0.28	0.86 ± 0.22	0.93 ± 0.12	0.97 ± 0.32
Hexyl acetate	0.03 ± 0.08	0.02 ± 0	0.01 ± 0	0.01 ± 0	0.02 ± 0
Ethyl octanoate	1.45 ± 0.05	0.84 ± 0.28	0.83 ± 0.16	1.13 ± 0.15	0.77 ± 0.17
Ethyl decanoate	1.79 ± 0.17	0.93 ± 0.07 (£)	0.86 ± 0.01 (£)	0.96 ± 0.02 (£)	0.92 ± 0.28 (£)
Diethyl succinate	0.73 ± 0.15	2.98 ± 1.02	6.45 ± 1.75 (*)	7.56 ± 1.56 (*)	12.11 ± 1.29 (*)
Ethyl dodecanoate	0.25 ± 0.08	0.26 ± 0.01	0.28 ± 0.02	0.29 ± 0.01	0.27 ± 0.02
<b>Other compounds</b>					
2,3-butanodione	1.33 ± 0.17	nd (£)	nd (£)	nd (£)	nd (£)
γ-butyrolactone	9.99 ± 0.21	nd (£)	6.91 ± 1.10	7.51 ± 0.50	7.55 ± 2.50
Furfural	0.09 ± 0.03	0.03 ± 0.005 (£)	0.04 ± 0 (£)	0.04 ± 0 (£)	0.04 ± 0 (£)

(\*): Significantly higher respect to control before MLF ( $P < 0.05$ ), (£): Significantly lower respect to control before MLF ( $P < 0.05$ ). nd.: not detected. Control wine correspond to non-inoculated wine.





**FIGURE 3** | Changes in the percentage of total alcohols (gray bars) and total esters (black bars) concentrations in the Malbec wine samples inoculated with single cultures of the strains UNQLp11, UNQLp155, UNQOe31b, and UNQOe73.2, and the blend cultures UNQLp11/UNQOe31b, UNQLp11/UNQOe73.2, UNQLp155/UNQOe31b, and UNQLp155/UNQOe73.2, or not inoculated (control). (£) indicates significantly lower respect to control before MLF; (\*) indicates significantly higher respect to control before MLF.

culture) respect to control wine. It is important to note that diethyl succinate was the only volatile compound that showed an increase in its concentration at day 14th with most of inoculated cultures, except with UNQLp11 and the blend UNQLp11/UNQOe31b. The single cultures UNQLp155, UNQOe31b and UNQOe73.2 showed values of diethyl succinate concentration of 5.46, 5.95 and 6.44 mg/L, respectively, at day 14, while blend cultures UNQLp11/UNQOe73.2, UNQLp155/UNQOe31b, and UNQLp155/UNQOe73.2 showed higher values, being these 6.45, 7.56 and 12.11 mg/L, respectively. It should be noted the remarkable synergistic effect showed by blend culture UNQLp155/UNQOe73.2.

On the other hand, all single or blend cultures were able to significantly reduce the furfural content in wine samples (Tables 1, 2). In relation to the volatile compound  $\beta$ -citronellol, it was detected in wine samples inoculated with the single cultures UNQLp11 and UNQOe73.2, but not when blend cultures of these strains were inoculated (Tables 1, 2). Finally, the presence of diacetyl (2,3-butanodione) could not be detected in any of the wine samples (Tables 1, 2).

## DISCUSSION

The results obtained in this work suggest that inoculation of a non-sterile Patagonian Malbec wine with single or blend cultures of selected native LAB strains, improves the performance of MLF in vinification assays at laboratory scale. Respect to consumption of L-malic acid, cultures containing *L. plantarum*

strains (as single or blend cultures) showed greater ability than those containing only *O. oeni* strains, a result that agrees with a better survival in wine of *L. plantarum* strains. The implantation analysis showed the presence of the inoculated strains after 14 days of incubation, but percentages of implantation were lower than 100%, suggesting that inoculated strains did not have an inhibitory effect on the wine natural microbiota. In addition, although changes in organic acid and volatile compounds profiles could have been due to the microbial community of the wine sample (natural microbiota and inoculated strains), the highest consumption of L-malic acid was only observed when wine was inoculated with LAB cultures.

Although many authors have studied the control and improvement of MLF by inoculation of single LAB starter cultures (Maicas et al., 1999; Ugliano et al., 2003; Pozo-Bayón et al., 2005; Costello et al., 2012; Garofalo et al., 2015; Iorizzo et al., 2016) and mixed *O. oeni* strains starter cultures (Carreté et al., 2006), as to our knowledge, no previous works have analyzed the effects of inoculating blend cultures of *O. oeni* and *L. plantarum* strains. Only one starter culture containing strains of these two LAB species is provided at commercial level, but these strains come from a different wine region that Argentinean North-Patagonia, and the employment of autochthonous strains of a specific wine-producing area has been strongly recommended by several author, as we mention in the introduction section (Ruiz et al., 2010; du Toit et al., 2011; Garofalo et al., 2015; Berbegal et al., 2016).

Several authors have reported that MLF improves wine flavor by reducing herbaceous notes, due to C6 alcohols content, and

enhances the fruity aroma, increasing esters content (Girard et al., 1997; Peinado et al., 2004; Costello et al., 2012; Feng et al., 2017). Inoculation of Malbec wine with autochthonous *L. plantarum* and *O. oeni* cultures, with the exception of those containing strain UNQLp11, showed an increase in esters content. While concentrations of some esters showed a decrease, such as ethyl butyrate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate, all Malbec wine samples showed values above the sensory threshold, which has been related to some fruity aromatic notes (strawberry, banana and apple odor) (Peinado et al., 2004; Feng et al., 2017). Additionally, a notable increase in diethyl succinate concentration was observed when wine samples were inoculated with single cultures of strains UNQLp155, UNQOe31b and UNQOe73.2 (5.46, 5.95 and 6.44 mg/L, respectively) and with blend cultures. Particularly, a synergistic effect was detected between strains UNQOe73.2 and UNQLp155 were inoculated as blend culture. An increase in diethyl succinate concentration is related to fruity and melon odor (Peinado et al., 2004) and was previously reported as a positive characteristic in several types of wines (Goldner et al., 2011; Knoll et al., 2012). Also, it has been described that reduction of furfural wine content, by LAB inoculation, contributes to diminish the caramel-like odor notes (Hale et al., 1999).

Strains UNQLp11 and UNQOe73.2 were also able to produce  $\beta$ -citronellol, which is an odorant terpene released by  $\beta$ -glucosidase activity, previously described in some LAB (Maturano and Saguir, 2017). Although this compound was below the sensory threshold, both strains seemed to have  $\beta$ -glucosidase activity that allowed aroma precursors hydrolysis and release of odorant aglycones, such as terpenes alcohols with pleasant floral odor properties. The development of new starter cultures able to improve the aromatic qualities of wine is required and further studies regarding this activity are needed.

On the other hand, it was shown that strain UNQLp155 was able to survive in Malbec wine during 14 days of MLF, whereas the viability of this strain in Pinot noir wine was lower (Brizuela et al., 2018). The difference in cell survival could be due to ethanol content of both wines [which is lower in Malbec wine (12.4% v/v) than in Pinot noir wine (14.5% v/v)], since ethanol can induce disruption of membrane cell integrity, and consequently a higher mortality rate (Bravo-Ferrada et al., 2014).

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## CONCLUSION

In conclusion, our results reveal a different malolactic behavior of single and blend LAB cultures. Regarding changes in the wine volatile compounds profile, *O. oeni* strains were able to produce higher amounts of significant volatile compounds due to their odor characteristics, indicating an active metabolism despite its lower viability compared to *L. plantarum* strains. The presence of *L. plantarum* strains in blend cultures guaranteed a higher consumption of L-malic acid, while *O. oeni* strains provide a greater capacity to change wine volatile compounds profile. The employ of such blend cultures to induce MLF in Malbec wines could offer an interesting advantage to improve the sensory attributes and quality of wine.

## AUTHOR CONTRIBUTIONS

NB and BB-F did the experimental work regarding malolactic fermentation (culturability, organic acid concentrations, and implantation of LAB strains). NB and MP-B did the determination of volatile compound profile and analyzed results obtained by HS-SPME and GC-MS technique. YC, LD, and AC did the experimental work regarding alcoholic fermentation. LS and ET coordinated the work (analysis of results, discussion, and writing of the manuscript). All authors have approved the final version of the manuscript.

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# Production of Food and Feed Additives From Non-food-competing Feedstocks: Valorizing *N*-acetylmuramic Acid for Amino Acid and Carotenoid Fermentation With *Corynebacterium glutamicum*

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*Corynebacterium glutamicum* is used for the million-ton-scale production of food and feed amino acids such as L-glutamate and L-lysine and has been engineered for production of carotenoids such as lycopene. These fermentation processes are based on sugars present in molasses and starch hydrolysates. Due to competing uses of starch and sugars in human nutrition, this bacterium has been engineered for utilization of alternative feedstocks, for example, pentose sugars present in lignocellulosic and hexosamines such as glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc). This study describes strain engineering and fermentation using *N*-acetyl-D-muramic acid (MurNAc) as non-food-competing feedstock. To this end, the genes encoding the MurNAc-specific PTS subunits MurP and Crr and the etherase MurQ from *Escherichia coli* K-12 were expressed in *C. glutamicum*  $\Delta nanR$ . While MurP and MurQ were required to allow growth of *C. glutamicum*  $\Delta nanR$  with MurNAc, heterologous Crr was not, but it increased the growth rate in MurNAc minimal medium from 0.15 h<sup>-1</sup> to 0.20 h<sup>-1</sup>. When in addition to *murP-murQ-crr* the GlcNAc-specific PTS gene *nagE* from *C. glycinophilum* was expressed in *C. glutamicum*  $\Delta nanR$ , the resulting strain could utilize blends of GlcNAc and MurNAc. Fermentative production of the amino acids L-glutamate and L-lysine, the carotenoid lycopene, and the L-lysine derived chemicals 1,5-diaminopentane and L-pipecolic acid either from MurNAc alone or from MurNAc-GlcNAc blends was shown. MurNAc and GlcNAc are the major components of the bacterial cell wall and bacterial biomass is an underutilized side product of large-scale bacterial production of organic acids, amino acids or enzymes. The proof-of-concept for valorization of MurNAc reached here has potential for biorefinery applications to convert non-food-competing feedstocks or side-streams to valuable products such as food and feed additives.

**Keywords:** L-lysine, diamino pentane, lycopene, L-glutamate, biorefinery, food additives, peptidoglycan, *N*-acetylmuramic acid

**Abbreviations:**  $\mu_{max}$ , maximal specific growth rate;  $\Delta S$ , variation of substrate concentration; gCDWL<sup>-1</sup>, gram of Cell Dry Weight per liter; q<sub>S</sub>, specific substrate uptake rate; Y<sub>P/S</sub>, yield coefficient of cell product per used substrate; Y<sub>P/X</sub>, yield coefficient of product per cell dry weight; Y<sub>X/S</sub>, yield coefficient of cell dry weight per used substrate.



## INTRODUCTION

*Corynebacterium glutamicum* is a predominantly aerobic, rod-shaped, Gram-positive soil bacterium which is generally recognized as safe (GRAS). Since the 1960s, *C. glutamicum* was first used for the production of the flavor enhancer (Kinoshita et al., 1957) under biotin limiting conditions (Shiio et al., 1962). *C. glutamicum* was developed into an important organism for the biotechnological industry, producing amino acids on a million-ton scale (Wendisch, 2014). *C. glutamicum* has also been engineered to produce diamines, organic acids, carotenoids, proteins and biopolymers (Wendisch et al., 2016). Recently, metabolic engineering of *C. glutamicum* to expand its substrate scope allowed to use alternative carbon sources that do not have competing uses in the food industry (Zahoor et al., 2012). Access to the hexosamines GlcN (Uhde et al., 2013) and GlcNAc (Matano et al., 2014) has been reported, but utilization of the hexosamine MurNAc as alternative carbon source by *C. glutamicum* has not been described (Dominguez et al., 1997; Cramer and Eikmanns, 2007).

GlcN and GlcNAc can be gained by hydrolysis of chitin and chitosan that make up the arthropod exoskeleton and are present in fungal cell walls. Every year, circa 100 billion tons of chitin are produced in Nature and GlcNAc and GlcN can be obtained by acid hydrolysis (Chen et al., 2010; Zhang and Yan, 2017) and are available, e.g., from shrimp shell waste, an abundant side stream of the fishery industry. MurNAc and GlcNAc are the hexosamine constituents of peptidoglycan which makes up about 5% of the cell mass of Gram-negative bacteria and up to 20% of the cell mass of Gram-positive bacteria (Munoz et al., 1967; Reith and Mayer, 2011). The peptidoglycan constituents that can be found in all bacterial habitats have been used as indicators of bacterial biomass content in soils (Domsch, 1982). The Gram-positive *C. glutamicum* is the main producing organism for the annual production of 5 million tons of amino acids (Wendisch et al., 2016). Under the assumptions that (a) the same amount of cell dry weight is produced, (b) 20% of the cell dry weight is peptidoglycan and (c) about half of peptidoglycan is GlcNAc and MurNAc, about 500.000 tons of MurNAc and GlcNAc would be available from the amino acid fermentation industry. Biotechnological processes with bacterial hosts are used at the million-ton scale to produce secreted compounds such as organic acids, amino acids and enzymes. The spent biomass may be used in waste-to-energy applications either by thermal (e.g., incineration), thermochemical (e.g., torrefaction) or by biochemical treatments (e.g., anaerobic digestion). However, it is desirable to make use of the carbon and nitrogen containing hexosamine fraction of peptidoglycan in food and feed fermentation processes. The hexosamines may function both as carbon and nitrogen source for bacterial fermentations.

In chemical hydrolysis, the hexosamine fraction of peptidoglycan is accessible via enzymes of bacterial cell wall recycling. The degradation of the own cell wall by autolytic enzymes as a part of cell wall recycling is a common pathway in bacteria. When an *Escherichia coli lys dap* mutant was labeled with [<sup>3</sup>H]diaminopimelate for two generations and

then chased, about 45% of its cell wall peptidoglycan was recycled per generation (Goodell and Schwarz, 1985; Uehara et al., 2006; Uehara and Park, 2008). The Gram-positive *Bacillus subtilis* can degrade, uptake and metabolize the cell wall component MurNAc in the stationary phase (Borisova et al., 2016). While all bacteria require cell wall peptidoglycan remodeling during growth and cell division, not all can utilize the monomeric components as carbon or nitrogen sources for growth. *C. glutamicum* may possess a minimal set of autolytic enzymes, however, many orthologs of the peptidoglycan degradation machinery from *E. coli* are absent (Dahl et al., 2004; Reith and Mayer, 2011). Catabolism of MurNAc in *E. coli* involves uptake and phosphorylation of MurNAc and GlcNAc via the phosphoenolpyruvate dependent phosphotransferase system (PTS). The MurNAc-specific PTS subunits are MurP and Crr (Figure 1). MurP, a two-domain protein that lacks a PTS-EIIA domain, is phosphorylated by EIIA<sup>Glc</sup>, a kinase encoded by the *crr* gene (carbohydrate repression resistance), which interacts with several members of the glucose PTS family (Nuoffer et al., 1988; Tchieu et al., 2001; Dahl et al., 2004). MurNAc-6-phosphate is further catabolized by the etherase MurQ (Figure 1) that cleaves the lactyl ether bond yielding GlcNAc-6-phosphate and D-lactate (Jaeger et al., 2005; Hadi et al., 2008). GlcNAc and GlcN are also taken up via the PTS with the specific subunits NagE (Plumbridge, 2009) and PTS<sup>Man</sup> (Curtis and Epstein, 1975). NagA deacetylates GlcNAc-6-phosphate to GlcN-6-phosphate which is deaminated by NagB to the glycolytic intermediate fructose-6-phosphate (Figure 1).

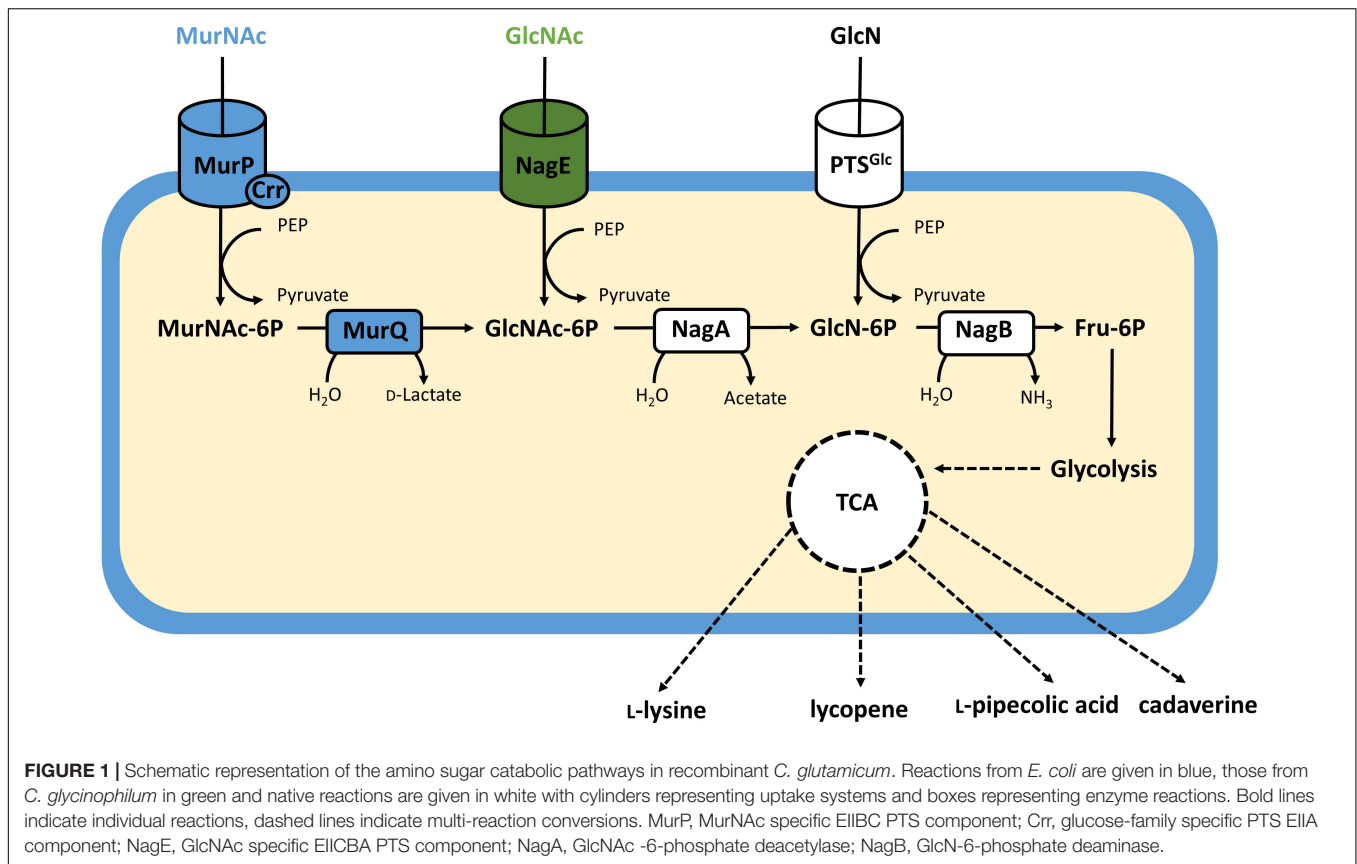
*Corynebacterium glutamicum* is able to take up GlcN (Figure 1) by using its glucose specific PTS PtsG (Arndt and Eikmanns, 2008; Uhde et al., 2013). Efficient growth with GlcN required high levels of the endogenous NagB, e.g., in the absence of the repressor protein NanR (Matano et al., 2016). By contrast, high levels of NagA and NagB were not sufficient to support growth with GlcNAc unless *nagE* from *C. glycinophilum* was expressed heterologously (Matano et al., 2014). Recombinant *C. glutamicum* strains carrying a *nanR* deletion and expressing *nagE* from *C. glycinophilum* produced several value-added products from GlcN or GlcNAc.

Here, *C. glutamicum* strains were developed for utilization of MurNAc as carbon, nitrogen and energy source and for MurNAc-based production of the food amino acid L-glutamate, the feed amino acid L-lysine, L-lysine-derived chemicals as well as the carotenoid lycopene.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

The strains and plasmids used in this work are listed in Table 1. Pre-cultivation of *C. glutamicum* strains was carried out at 30°C in baffled shake flasks using BHI supplemented with 45.5 g/L D-sorbitol. *E. coli* was grown at 37°C in LB (Lysogeny Broth) medium. Kanamycin (50 µg/mL), chloramphenicol (4.5 and 25 µg/mL for *C. glutamicum* and *E. coli*, respectively) or tetracycline (5 µg/mL) were added, if necessary. To adjust both cultures to growth conditions in the Biolector® system (m2pLabs,



Baesweiler), precultures were washed after 24 h with TN-buffer and transferred to CGXII medium (Eggeling and Bott, 2005) with 100 mM GlcN and antibiotics, if necessary. After 24 h, these were transferred to 1 mL cultures in the Biolector® system (1100 rpm) with CGXII medium containing and, if not otherwise stated, 25 mM MurNAc (BACHEM, Bubendorf, Switzerland) as sole carbon source or a combination of 25 mM MurNAc and 25 mM GlcNAc. To trigger glutamate production, penicillin G (10 μM) was added in the main culture. The initial OD<sub>600</sub> was 1 and gene expression from plasmids pVWEx1 and pEC-XT99A was induced by addition of 25 μM IPTG, if not otherwise stated. Correlation factors for light scattering in the Biolector system, OD<sub>600</sub> and biomass concentrations were determined.

## Construction of Expression Vectors

*Escherichia coli* DH5α was used for cloning. Codon usage of *murP*, *crr* and *murQ* from *E. coli* for *C. glutamicum* was examined using the graphical codon usage analyzer<sup>1</sup>. The analysis showed, that the codon ATA occurred twice in the sequence of *murP*. This codon is rarely used in *C. glutamicum* and was changed to the more frequently used codon of ATC via site directed mutagenesis (SDM). The mutated variation of *murP* was called *murP*<sup>opt</sup>. The genes of *murP*, *crr* and *murQ* were amplified via PCR from genomic DNA of *E. coli* K-12, while *nagE* from *C. glycinophilum*, was amplified from

pVWEx1\_ *nagE* (Matano et al., 2016). The primers used in this study (see **Supplementary Table S1**) were obtained from Metabion international AG, Planegg. Using Gibson assembly (Gibson, 2011), the vectors pVWEx1\_ *murP*, pVWEx1\_ *murP*<sup>opt</sup>, pVWEx1\_ *murP**crr*, pVWEx1\_ *murP*<sup>opt</sup>*crr*, pCXE50\_ *murQ* and pEC-XT99A\_ *nagE* were constructed. The vectors pVWEx1 and pEC-XT99A are IPTG inducible while pCXE50 has a constitutive EF<sub>tu</sub> promoter. *E. coli* was transformed by the CaCl<sub>2</sub> method while transformation through electroporation was applied for *C. glutamicum* at 2500 V, 25 μF and 200 Ω.

## Carotenoid Extraction

Lycopene was extracted as described before (Heider et al., 2012). In short, 5 wells each containing 1 ml cell suspension were combined and pelleted in safe lock micro reaction tubes by centrifugation at 10,000 g for 15 min and resuspended in 800 μL of a 7:3 methanol/acetone mixture and incubated for 15 min at 60°C and 750 rpm in a thermomixer (Eppendorf). The cell debris was removed by centrifugation and the supernatant used for HPLC analysis. The procedure was repeated to ensure complete extraction until white pellets were obtained.

## Quantitation of Fermentation Products

The quantification of MurNAc, GlcNAc, lycopene, L-glutamate, and L-lysine was conducted by HPLC analysis (1200 series

<sup>1</sup><http://gcua.schoedl.de/>

**TABLE 1** | Plasmids and strains used in this study.

Strain ID	Characteristics	Reference
<b><i>E. coli</i></b>		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>dlacZ</i> $\Delta$ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan, 1983
JW2421-1	F-, $\Delta$ ( <i>araD-araB</i> )567, $\Delta$ <i>lacZ4787</i> (: <i>rrmB-3</i> ), $\lambda$ -, $\Delta$ <i>murQ757</i> : <i>kan</i> , <i>rph-1</i> , $\Delta$ ( <i>rhaD-rhaB</i> )568, <i>hsdR514</i> ; Kan <sup>R</sup>	Baba et al., 2006
JW2421-1 (pCXE50_ <i>murQ</i> )	JW2421-1 carrying pCXE50_ <i>murQ</i>	this work
<b><i>C. glutamicum</i></b>		
$\Delta$ <i>nanR</i>	ATCC 13032 $\Delta$ <i>nanR</i>	(Matano et al., 2016)
$\Delta$ <i>nanR</i> PQ	$\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> and pCXE50_ <i>murQ</i>	This work
$\Delta$ <i>nanR</i> POQ	$\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> <sup>opt</sup> and pCXE50_ <i>murQ</i>	This work
$\Delta$ <i>nanR</i> PCQ	$\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> and pCXE50_ <i>murQ</i>	This work
$\Delta$ <i>nanR</i> POCQ	$\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> <sup>opt</sup> _ <i>crr</i> and pCXE50_ <i>murQ</i>	This work
$\Delta$ <i>nanR</i> PCQnE	$\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>nagE</i>	This work
DM1729 $\Delta$ <i>nanR</i> PCQ	DM1729 $\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> and pCXE50_ <i>murQ</i>	This work
DM1729 $\Delta$ <i>nanR</i>	ATCC 13032 <i>pycP</i> <sup>458S</sup> <i>hom</i> <sup>V59A</sup> <i>lysC</i> <sup>T311I</sup> $\Delta$ <i>nanR</i> , L-lysine overproducing strain	Matano et al., 2014
DM1729 $\Delta$ <i>nanR</i> PCQnE	DM1729 $\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>nagE</i>	This work
DM1729 $\Delta$ <i>nanR</i> PCQ <i>ldcC</i>	DM1729 $\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>ldcC</i>	This work
DM1729 $\Delta$ <i>nanR</i> PCQ LPA	DM1729 $\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> , pCXE50_ <i>murQ</i> and pECXT99A_ <i>lysDH</i> <sub>S.prom</sub> - <i>proC</i> <sub>C.g</sub>	This work
$\Delta$ <i>crtY</i> Eb $\Delta$ <i>nanR</i>	lycopene producing derivative of WT carrying in-frame $\Delta$ <i>crtY</i> Eb $\Delta$ <i>nanR</i>	Matano et al., 2014
$\Delta$ <i>crtY</i> Eb $\Delta$ <i>nanR</i> PCQ	Deletion of <i>crtY</i> and <i>crtEb</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> , pCXE50_ <i>murQ</i>	This work
$\Delta$ <i>crtY</i> Eb $\Delta$ <i>nanR</i> PCQnE	$\Delta$ <i>crtY</i> Eb $\Delta$ <i>nanR</i> carrying pVWEx1_ <i>murP</i> _ <i>crr</i> , pCXE50_ <i>murQ</i> and pEC-XT99A_ <i>nagE</i>	This work
<b>Plasmids</b>		
pVWEx1	<i>E. coli</i> / <i>C. glutamicum</i> shuttle vector, Kan <sup>R</sup>	Peters-Wendisch et al., 2001
pEC-XT99A	Tet <sup>R</sup> , <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector (Ptrc, <i>lacI</i> , pGA1 OriV <sub>C.g</sub> .)	Kirchner and Tauch, 2003
pCXE50_ <i>porB</i>	Constitutive pE <sub>f<sub>lu</sub></sub> promoter, Cm <sup>R</sup>	Lee, 2014
pVWEx1_ <i>murP</i>	pVWEx1-derivative for IPTG inducible expression of <i>murP</i> from <i>E. coli</i> , Kan <sup>R</sup>	This work
pVWEx1_ <i>murP</i> <sup>opt</sup>	pVWEx1-derivative for IPTG inducible expression of the for <i>C. glutamicum</i> codon optimized <i>murP</i> from <i>E. coli</i> , Kan <sup>R</sup>	This work
pVWEx1_ <i>murP</i> _ <i>crr</i>	pVWEx1-derivative for IPTG inducible expression of <i>murP</i> and <i>crr</i> from <i>E. coli</i> , Kan <sup>R</sup>	This work
pVWEx1_ <i>murP</i> <sup>opt</sup> _ <i>crr</i>	pVWEx1-derivative for IPTG inducible expression of the for <i>C. glutamicum</i> codon optimized <i>murP</i> and <i>crr</i> from <i>E. coli</i> , Kan <sup>R</sup>	This work
pCXE50_ <i>murQ</i>	Cm <sup>R</sup> , expressing <i>murQ</i> from <i>E. coli</i> K12	This work
pEC-XT99A_ <i>nagE</i>	Tet <sup>R</sup> , expressing <i>nagE</i> from <i>Corynebacterium glycinophilum</i> DSM45794	This work
pEC-XT99A_ <i>ldcC</i>	Tet <sup>R</sup> , expressing <i>ldcC</i> from <i>E. coli</i> K12	This work
pECXT99A_ <i>lysDH</i> <sub>S.prom</sub> - <i>proC</i> <sub>C.g</sub>	Tet <sup>R</sup> , expressing <i>lysDH</i> from <i>Silicibacter pomeroyi</i> and <i>proC</i> from <i>C. glutamicum</i>	This work

Kan<sup>R</sup>, kanamycin resistance; Tet<sup>R</sup>, tetracycline resistance; Cm<sup>R</sup>, chloramphenicol resistance.

HPLC system, Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn). The supernatant of 1 ml pelleted cell suspension was diluted and analyzed. For quantification of organic acids, the carbo column (300 × 8 mm, 10  $\mu$ m particle size, 25 Å pore diameter, CS Chromatographie Service GmbH) and a refractive index detector (RID G1362A, 1200 series, Agilent Technologies) was used for quantification of MurNAc and GlcNAc with 5 mM H<sub>2</sub>SO<sub>4</sub> as buffer.

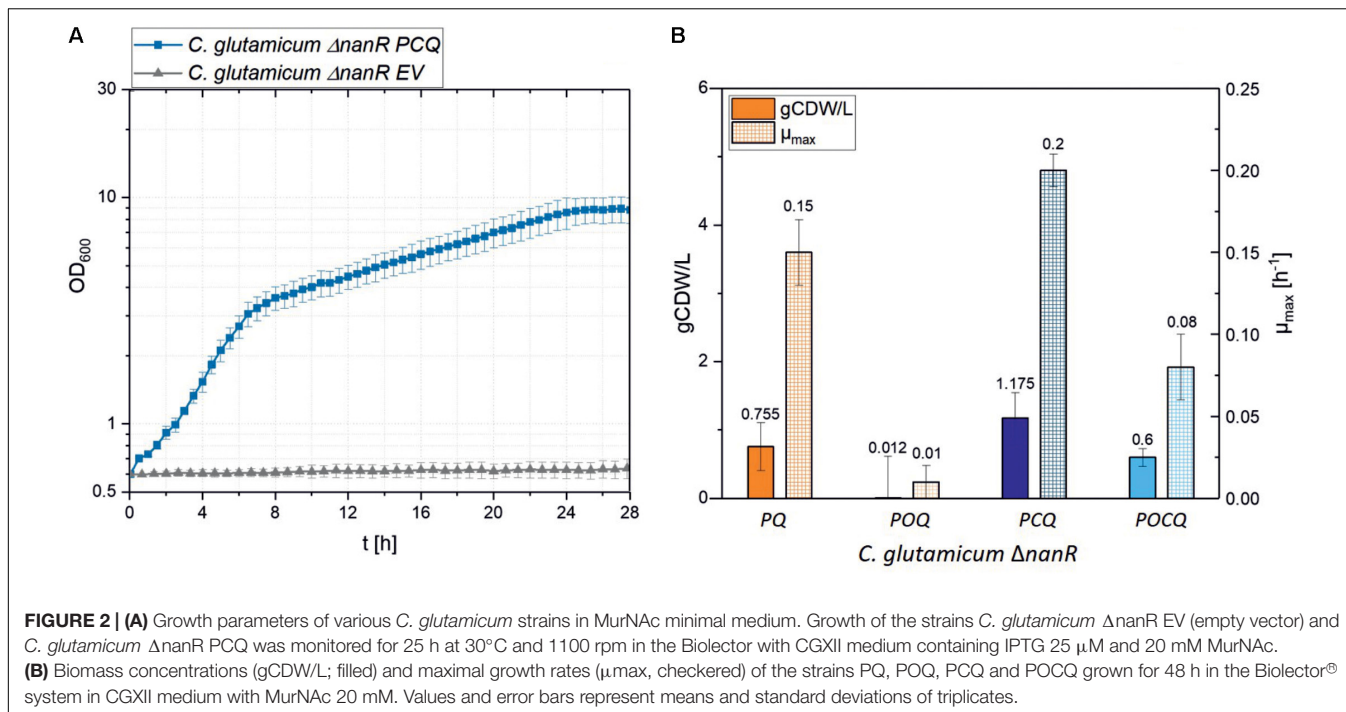
Applying OPA derivatisation, L-lysine and L-glutamate were analyzed using an RP8 column with a sodium acetate (0.25 v/v %) buffer at pH 6 and a 1:50 dilution with an internal L-asparagine

standard. Using the RP18 column with a Methanol-Milli-Q-water mixture (9:1), lycopene was quantified (Heider et al., 2012).

## RESULTS

### Metabolic Engineering of *C. glutamicum* for Growth With MurNAc as Carbon Source

*Corynebacterium glutamicum*, which has been engineered to utilize GlcN and GlcNAc (Matano et al., 2014, 2016), cannot



utilize MurNac since no growth was observed in minimal medium with 25 mM MurNac and  $25 \pm 0.1$  mM MurNac remained in the growth medium after 25 h of incubation (Figure 2A). As expected, the *C. glutamicum* genome lacks genes encoding a MurNac PTS and MurNac-6-phosphate etherase for uptake and conversion of MurNac to GlcNac-6-phosphate, an endogenous intermediate of *C. glutamicum* metabolism. As described in Section “Materials and Methods,” the genes for the MurNac PTS *murP* from *E. coli* or codon optimized allele *murP<sup>opt</sup>* (Nuoffer et al., 1988; Tchieu et al., 2001; Dahl et al., 2004) were cloned into the IPTG-inducible plasmid pVWex1 alone or as operon with *crr*. The gene for the MurNac-6-phosphate etherase *murQ* from *E. coli* (Jaeger et al., 2005) was cloned into the constitutive expression vector pCXE50 (Lee, 2014). Functional expression of *murQ* from pCXE50\_ *murQ* was tested by complementation of the *E. coli murQ* mutant *E. coli* JW2421-1. While *E. coli* JW2421-1(pCXE50\_ *murQ*) utilized MurNac as sole carbon source ( $\Delta OD_{600}$  of  $3.2 \pm 0.1$  and  $\mu_{max}$  of  $0.07 \pm 0.01$  h<sup>-1</sup>), *E. coli* JW2421-1 $\Delta murQ$  showed no growth (see Supplementary Figure S1). Only *murQ* was tested by complementation of a *E. coli* mutant. We neither tested *murP* nor *crr* because we expected perturbations due to overexpression since MurP is a membrane protein and Crr serves a regulatory function. *C. glutamicum*  $\Delta nanR$  was transformed with the constructed pVWex1 plasmids and with the pCXE50\_ *murQ*. The respective strains were named *C. glutamicum*  $\Delta nanR$  PQ, POQ, PCQ, POCQ (Table 1).

Strains expressing *crr* from *E. coli* grew faster in minimal medium containing 25 mM MurNac as sole source of carbon and energy than strains lacking *crr* (Figure 2B). Strains with native *murP* grew better than strains expressing codon optimized *murP*

(Figure 2B). IPTG was used at a low concentration (25  $\mu$ M) to induce heterologous gene expression, since higher concentrations slowed growth (Table 2). This is not unexpected and presumably due to too high expression of transport protein genes as seen previously for *dccT* (Youn et al., 2008) and *dctA* (Youn et al., 2009), coding for dicarboxylate transporters. With 25  $\mu$ M IPTG, strain  $\Delta nanR$  PCQ expressing native *murP*, *crr* and *murQ* grew in minimal medium containing 25 mM MurNac to a biomass concentration of  $1.2 \pm 0.3$  gCDW/L and with 50 mM MurNac to a biomass concentration of  $2.0 \pm 0.2$  gCDW/L (Table 2). Biphasic exponential growth was observed: faster growth between 0 and 6 h and slower growth between 6 and 27 h (Figure 2A). The curves appear linear as the Y axis has been logarithmized (Figure 2). During the transition from the first to the second growth phase the medium contained  $3.10 \pm 0.12$  mM lactate. Thus, lactate released by MurQ from MurNac-6-phosphate may not have been utilized as fast as GlcNac-6-phosphate, the other product of the MurQ reaction. In consequence, lactate accumulated in the culture medium in the first exponential growth phase and presumably slowed growth in the second exponential growth phase. Transient accumulation of lactate to growth inhibitory concentrations has been observed during growth of *C. glutamicum* with various carbon sources (Engels et al., 2008).

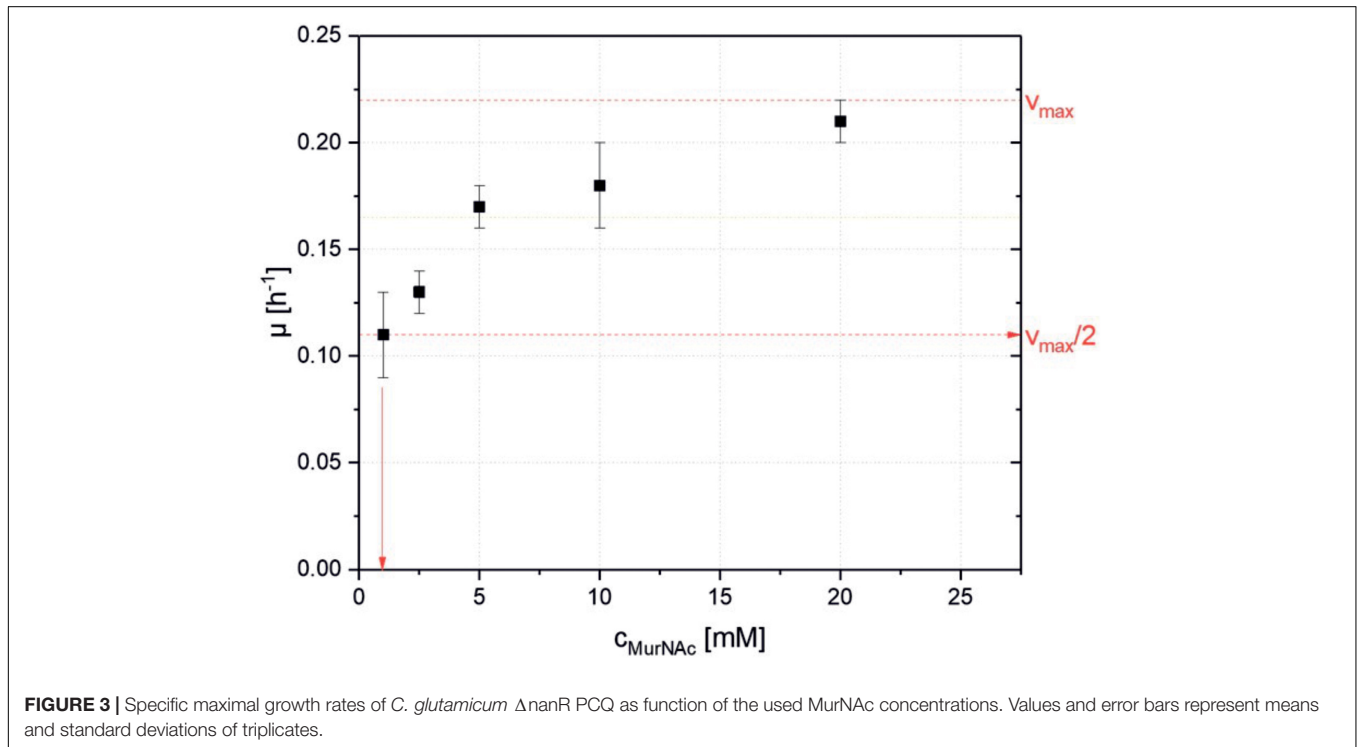
As PTS systems typically support growth on their cognate substrates with high affinity, the dependence of the growth rate on the initial MurNac concentration in the growth medium was determined using strain  $\Delta nanR$  PCQ. Different concentrations of MurNac (1, 2.5, 5, 10, and 20 mM) were used and the maximal growth rates were plotted against the MurNac concentration to derive the maximal growth rate of  $0.22$  h<sup>-1</sup> and the Monod constant of  $0.9 \pm 0.1$  mM as shown in Figure 3.



**TABLE 2** | Growth characteristics of *C. glutamicum*  $\Delta nanR$  PCQ on different MurNAc and IPTG concentrations.

$C_{MurNAc}$ [mM]	$C_{IPTG}$ [ $\mu$ M]	gCDW/L	$\mu_{max1}$ [ $h^{-1}$ ]	$\mu_{max2}$ [ $h^{-1}$ ]	$Y_{x/s}$ [ $g \cdot mol^{-1}$ ]
20	25	1.2 $\pm$ 0.3	0.25 $\pm$ 0.01	0.05 $\pm$ 0.01	58.0 $\pm$ 0.1
	50	1.0 $\pm$ 0.4	0.24 $\pm$ 0.01	0.04 $\pm$ 0.01	51.4 $\pm$ 0.1
	100	1.0 $\pm$ 0.1	0.26 $\pm$ 0.02	0.05 $\pm$ 0.01	54.5 $\pm$ 0.1
50	25	2.0 $\pm$ 0.2	0.25 $\pm$ 0.04	0.04 $\pm$ 0.01	38.4 $\pm$ 0.1
	50	1.7 $\pm$ 0.5	0.26 $\pm$ 0.01	0.05 $\pm$ 0.01	35.1 $\pm$ 0.1
	100	1.5 $\pm$ 0.2	0.21 $\pm$ 0.02	0.04 $\pm$ 0.01	30.4 $\pm$ 0.1

The final biomass concentrations reached [given as g of cell dry weight (CDW) per L], maximal specific growth rates of the first ( $\mu_{max1}$ ) and second growth phase ( $\mu_{max2}$ ), and the biomass yield coefficients of cell dry weight formed per used substrate used ( $Y_{x/s}$ ) are given as means with standard deviations.



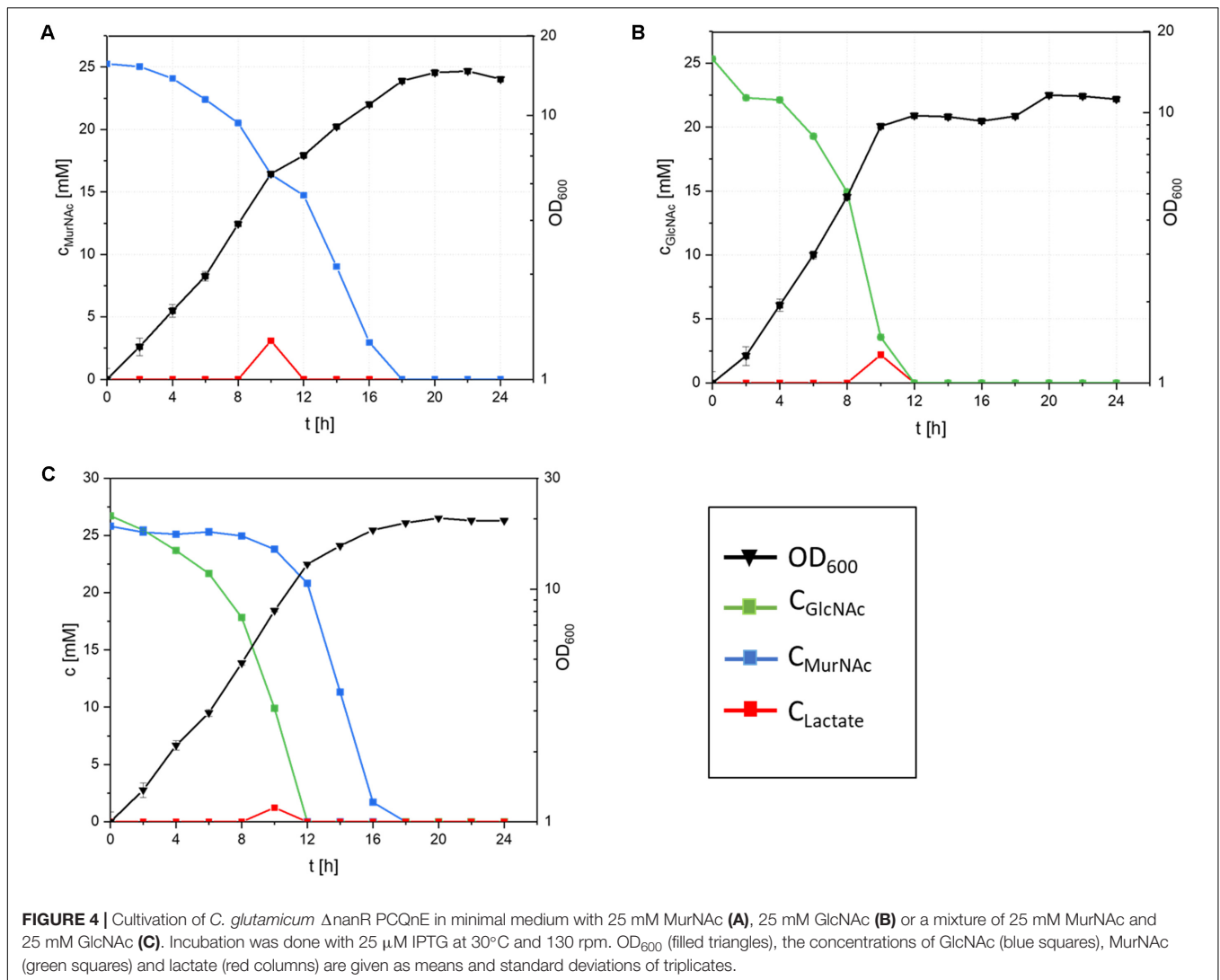
A sub-millimolar Monod constant is typical for PTS mediated uptake.

### Comparative Analysis of Growth With MurNAc and/or GlcNAc

Growth of recombinant *C. glutamicum* with MurNAc and/or GlcNAc as sole carbon sources was compared (Figure 4 and Table 3). With 25 mM MurNAc *C. glutamicum*  $\Delta nanR$  PCQnE grew to a biomass concentration of 3.0  $\pm$  0.1 gCDW/L, while the maximal biomass concentration was only 2.4  $\pm$  0.1 gCDW/L with GlcNAc. The higher biomass concentration observed with MurNAc in comparison to GlcNAc indicated that lactate released from MurNAc by MurQ contributed to biomass formation. However, the biomass yield was higher with GlcNAc (0.44  $\pm$  0.01  $g \cdot g^{-1}$ ) than with MurNAc (0.39  $\pm$  0.02  $g \cdot g^{-1}$ ). GlcNAc catabolism was faster than MurNAc catabolism as the maximal growth rates and the specific substrate uptake rates were lower with MurNAc (0.22  $\pm$  0.10  $h^{-1}$  and

1.80  $\pm$  0.10  $mmol \cdot g^{-1} \cdot h^{-1}$ ) than with GlcNAc (0.30  $\pm$  0.01  $h^{-1}$  and 3.00  $\pm$  0.10  $mmol \cdot g^{-1} \cdot h^{-1}$ ) as shown in Table 3.

Unlike *E. coli* and *B. subtilis*, it is typical for *C. glutamicum* to simultaneously co-utilize carbon substrates present in blends (Blombach and Seibold, 2010). Therefore, *C. glutamicum* strain  $\Delta nanR$  PCQnE was constructed by transforming strain  $\Delta nanR$  PCQnE with plasmid pEC-XT99A-*nagE* for expression of the gene for the GlcNAc-specific PTS uptake system to establish whether MurNAc and GlcNAc are co-utilized or utilized sequentially. *C. glutamicum* strain  $\Delta nanR$  PCQnE were grown with 25 mM MurNAc and/or 25 mM GlcNAc (Figure 4). With the blend of MurNAc and GlcNAc *C. glutamicum*  $\Delta nanR$  PCQnE grew to a biomass concentration of 3.8  $\pm$  0.1 gCDW/L, while a biomass concentration of only 2.1  $\pm$  0.1 g/L was reached in the absence of *nagE*. Determination of the residual substrate concentrations revealed sequential utilization of GlcNAc before MurNAc (Figure 4C). Thus, unlike many growth substrates MurNAc and GlcNAc were not co-utilized.



**FIGURE 4 |** Cultivation of *C. glutamicum*  $\Delta nanR$  PCQnE in minimal medium with 25 mM MurNAc (A), 25 mM GlcNAc (B) or a mixture of 25 mM MurNAc and 25 mM GlcNAc (C). Incubation was done with 25  $\mu$ M IPTG at 30°C and 130 rpm.  $OD_{600}$  (filled triangles), the concentrations of GlcNAc (blue squares), MurNAc (green squares) and lactate (red columns) are given as means and standard deviations of triplicates.

**TABLE 3 |** Cultivation parameters of *C. glutamicum*  $\Delta nanR$  PCQnE growing on MurNAc (25 mM), GlcNAc (25 mM) or a MurNAc-GlcNAc-mixture (both 25 mM).

	MurNAc	GlcNAc	MurNAc-GlcNAc mix
$\Delta OD_{600}$ [–]	$12.0 \pm 0.1$	$9.6 \pm 0.1$	$17.7 \pm 0.1$
$\Delta S$ MurNAc [mM]	$25.3 \pm 0.1$	–	$25.8 \pm 0.1$
$\Delta S$ GlcNAc [mM]	–	$25.3 \pm 0.1$	$26.7 \pm 0.1$
$Y^1_{X/S}$ [g·mmol <sup>-1</sup> ]	$0.14 \pm 0.01$	$0.09 \pm 0.01$	$0.11 \pm 0.10$
$\mu_{max}^1$ [h <sup>-1</sup> ]	$0.18 \pm 0.01$	$0.22 \pm 0.01$	$0.18 \pm 0.01$
$qS^1$ [mmol·g <sup>-1</sup> ·h <sup>-1</sup> ]	$1.27 \pm 0.10$	$2.42 \pm 0.10$	$1.68 \pm 0.10$
$Y^2_{X/S}$ [g·mol <sup>-1</sup> ]	$0.11 \pm 0.01$	–	$0.08 \pm 0.10$
$\mu_{max}^2$ [h <sup>-1</sup> ]	$0.10 \pm 0.01$	–	$0.07 \pm 0.20$
$qS^2$ [mmol·g <sup>-1</sup> ·h <sup>-1</sup> ]	$0.87 \pm 0.10$	–	$0.90 \pm 0.20$

The parameters given are  $\Delta OD_{600}$  for biomass formed and  $\Delta S$  for substrate used. Monophasic growth was observed with GlcNAc. With MurNAc, two phases were observed, and biomass yield coefficients of cell dry weight formed per used substrate used ( $Y_{X/S}$ ), maximal specific growth rates ( $\mu_{max}$ ) and specific substrate uptake rates ( $qS$ ) are given for the phase 1 and phase 2. With MurNAc+GlcNAc,  $Y_{X/S}$ ,  $\mu_{max}$  and  $qS$  are reported for the phase where GlcNAc was utilized exclusively (phase 1; 0–8 h) as well as for the phase when MurNAc was utilized exclusively (phase 2; 12–18 h).

**TABLE 4** | Parameters describing concentration in mM and production yield ( $Y_{P/S}$ ) of L-lysine, L-PA and 1,5-diaminopentane after 72 h with either 25 mM GlcNAc, either 25 mM MurNAc or 25mM GlcNAc of indicated strains and glutamate production after 48 h from either 25 mM MurNAc either a mixture of GlcNAc and MurNAc, each 25 mM.

Product	Strain ID	C <sub>Substrate</sub> [mM]	C <sub>product</sub> [mM]	Y <sub>P/S</sub> [mmol·mmol <sup>-1</sup> ]
Lycopene	$\Delta crtYEB \Delta nanR PCQ$	25 mM MurNAc	–	0.04 ± 0.01 mg (gCDW) <sup>-1</sup>
	$\Delta crtYEB \Delta nanR PCQnE$	25mM GlcNAc + 25 mM MurNAc		0.10 ± 0.01 mg (gCDW) <sup>-1</sup>
L-lysine	DM1729 $\Delta nanR PCQnE$	25 mM GlcNAc	7.6 ± 0.3	0.30 ± 0.10
	DM1729 $\Delta nanR PCQnE$	25 mM GlcNAc + 25 mM MurNAc	10.6 ± 0.6	0.21 ± 0.10
	DM1729 $\Delta nanR$	25mM MurNAc	6.9 ± 0.6	0.27 ± 0.05
1,5-diaminopentane	DM1729 $\Delta nanR PCQ ldcC$	25 mM MurNAc	4.3 ± 0.1	0.30 ± 0.10
L-PA	DM1729 $\Delta nanR PCQ LPA$	25 mM MurNAc	4.2 ± 0.2	0.35 ± 0.10
Glutamate	$\Delta nanR PCQ$	25 mM MurNAc	1.0 ± 0.1	0.03 ± 0.00
	$\Delta nanR PCQnE$	25mM GlcNAc + 25 mM MurNAc	1.9 ± 0.1	0.04 ± 0.00

## MurNAc-Based Production of Food and Feed Additives and Derived Chemicals

MurNAc was expected not only to support growth of recombinant *C. glutamicum* strains, but also production of value-added compounds. Therefore, MurNAc was tested as sole carbon source or in blends with GlcNAc for production of the amino acids L-lysine and L-glutamate, the diamine 1,5-diaminopentane, the cyclic non-proteinogenic amino acid L-pipecolic acid, and the carotenoid lycopene.

The lycopene accumulating strain *C. glutamicum*  $\Delta crtYEB \Delta nanR$  (Matano et al., 2014) was transformed with the plasmids pVWEx1<sub>murP\_crr</sub>, pEC-XT99A<sub>nagE</sub> and pCXE50<sub>murQ</sub> as described above and the resulting strains were named  $\Delta crtYEB \Delta nanR PCQ$  and  $\Delta crtYEB \Delta nanR PCQnE$ . Cells of both strains accumulated lycopene when grown in MurNAc containing minimal medium. Strain  $\Delta crtYEB \Delta nanR PCQ$  showed a lycopene content of 0.04 mg ± 0.01 (g CDW)<sup>-1</sup> in MurNAc minimal medium. Growth of *C. glutamicum*  $\Delta crtYEB \Delta nanR PCQnE$  with a MurNAc/GlcNAc blend led to a lycopene content of 0.10 ± 0.01 mg (g CDW)<sup>-1</sup>.

The L-lysine producing strains *C. glutamicum* DM1729 $\Delta nanR PCQ$  and DM1729 $\Delta nanR PCQnE$  were constructed based on DM1729 $\Delta nanR$  as described above for lycopene accumulating strains. DM1729 $\Delta nanR PCQ$  produced 7 ± 1 mM L-lysine ( $Y_{P/S}$  0.27 ± 0.05 mmol mmol<sup>-1</sup>) and DM1729 $\Delta nanR PCQnE$  produced 11 ± 1 mM L-lysine ( $Y_{P/S}$  0.21 ± 0.10 mmol mmol<sup>-1</sup>) in minimal medium with either 25 mM MurNAc or a combination of 25 mM MurNAc and 25 mM GlcNAc., whereas 7.6 ± 0.3 mM L-lysine ( $Y_{P/S}$  0.30 ± 0.01 mmol mmol<sup>-1</sup>) have been produced from DM1729PCQnE with 25 mM GlcNAc (Table 4).

To test if the L-lysine derived compounds 1,5-diaminopentane and L-pipecolic acid can also be produced from MurNAc, strain DM1729 $\Delta nanR PCQ$  was transformed with either pEC-XT99A<sub>ldcC</sub> or pEC-XT99A<sub>lysDH-proC</sub>. 1,5-Diaminopentane can be generated from L-lysine by lysine decarboxylase LdcC and L-pipecolic acid can be generated from L-lysine in a three-step pathway by L-lysine-6-dehydrogenase (encoded by

*lysDH* from *S. pomeroyi*), spontaneous ring formation and by pyrroline 5-carboxylate reductase (encoded by endogenous *proC*) (Pérez-García et al., 2016, 2017). Although the strains showed poor growth, *C. glutamicum*  $\Delta nanR$  DM1729PCQ *ldcC* was able to produce 4.3 ± 0.1 mM of 1,5-diaminopentane ( $Y_{P/S}$  0.30 ± 0.10 mmol mmol<sup>-1</sup>) and *C. glutamicum*  $\Delta nanR$  DM1729PCQ *LPA* produced 4.0 ± 0.2 mM of L-pipecolic acid ( $Y_{P/S}$  0.35 ± 0.10 mmol mmol<sup>-1</sup>) from MurNAc as sole carbon source as shown in Table 4.

L-Glutamate production was accomplished by *C. glutamicum*  $\Delta nanR PCQ$  and *C. glutamicum*  $\Delta nanR PCQnE$  using penicillin G as trigger. *C. glutamicum*  $\Delta nanR PCQ$  accumulated 1 ± 0 mM of L-glutamate from 25 mM MurNAc after 48 h, whereas *C. glutamicum*  $\Delta nanR PCQnE$  produced 2 ± 0 mM of L-glutamate under these conditions.

## DISCUSSION

In this work, production of food and feed additives by *C. glutamicum* from MurNAc, an alternative carbon source without competing use in human and animal nutrition, has been established. The food amino acid L-glutamate, the feed amino acid L-lysine and the feed additive lycopene were produced from MurNAc, GlcNAc and blends of both hexosamines.

Metabolic engineering of *C. glutamicum* for access to MurNAc relied on *E. coli* genes. Its MurNAc PTS system was active in *C. glutamicum* and strains expressing *crr* in addition were able to grow faster and yield more biomass than the strains without heterologous Crr (Figure 2). Crr is a glucose-family specific EIIC component, but it can interact with the PTS-EIIBC components of several members of the glucose PTS family (Barabote and Saier, 2005). *C. glutamicum* has two complete PTS<sup>Glc</sup> systems (Barabote and Saier, 2005). The finding that MurNAc could be utilized without heterologous Crr indicated that a PTS component of *C. glutamicum* made up for its absence. Moreover, the low Monod constant found for growth of the recombinant with MurNAc (Figure 3) indicated

that the MurNAc PTS catalyzed high-affinity MurNAc uptake in *C. glutamicum*, although the MurNAc PTS seems to have a lower affinity for its substrate than, e.g., the heterologous expressed GlcNAc specific PTS system NagE from *Corynebacterium glycinophilum* which showed a  $K_M$  value  $3.8 \pm 0.6 \mu\text{M}$  (Ferenci, 1996; Matano et al., 2014). The  $K_M$  for the etherase MurQ in *E. coli* found in literature had a similar range of value (1.2 mM) (Hadi et al., 2008) with the  $K_M$  value found experimentally in this study for the MurNAc PTS ( $0.9 \pm 0.1 \text{ mM}$ ) system, making the two enzymatic steps of up taking, phosphorylation and esterification balanced.

Growth of recombinant *C. glutamicum* with MurNAc was biphasic and lactate accumulated during the interphase (Figure 4A). MurNAc differs from GlcNAc only by one additional lactoyl group that is hydrolysed to lactate by etherase MurQ. Although *C. glutamicum* can utilize lactate as sole carbon source (Eikmanns, 2005), lactate accumulated. Utilization of D-lactate requires *dld* encoding quinone-dependent D-lactate dehydrogenase (Stansen et al., 2005; Kato et al., 2010). Utilization of L-lactate requires quinone-dependent L-lactate dehydrogenase which is encoded in the *LldR* repressed operon *cg3227-lldD* (Engels et al., 2008; Georgi et al., 2008). L-Lactate is secreted by *C. glutamicum* under certain conditions, e.g., during growth with glucose when oxygen is limiting, but is quickly re-utilized once *cg3227-lldD* is derepressed (Eggeling and Bott, 2005; Stansen et al., 2005; Georgi et al., 2008).

*Corynebacterium glutamicum* co-utilizes glucose simultaneously with many different carbon sources including those that required introduction of heterologous pathways, for example, xylose (requiring xylose isomerase gene from *E. coli* (Kawaguchi et al., 2006), arabinose (requiring the *araBAD* operon from *E. coli* (Kawaguchi et al., 2008; Schneider et al., 2011), cellobiose (requiring  $\beta$ -glucosidase) or glycerol (requiring *E. coli* glycerol kinase and glycerol-3-phosphate dehydrogenase), (Rittmann et al., 2008; Sasaki et al., 2008; Zahoor et al., 2012; Meiswinkel et al., 2013; Wendisch et al., 2016). Only rarely, glucose repression has been observed, for example, during sequential utilization of glucose before ethanol (due to catabolite repression of the alcohol dehydrogenase gene *adhA*) (Gerstmeir et al., 2004) or before glutamate (due to catabolite repression of the operon *gluABCD* encoding the glutamate uptake system) (Wendisch et al., 2000; Blombach and Seibold, 2010). The preferential utilization of GlcNAc before MurNAc by *C. glutamicum*  $\Delta nanR$  *PCQnE* may be explained by an offset between fast uptake and hydrolysis of MurNAc yielding GlcNAc-6-P and D-lactate followed by fast utilization of GlcNAc-6-P, but accumulation of D-lactate. It is conceivable that overexpression of *dld* would accelerate D-lactate catabolism precluding transient D-lactate accumulation during growth with MurNAc.

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A proof-of-concept for MurNAc-based fermentative production of food and feed additives was reached. The engineered strain DM1729 $\Delta nanR$  *PCQ* and DM1729 $\Delta nanR$  *PCQnE* showed comparable L-lysine production as observed previously for GlcN and GlcNAc (Matano et al., 2014). Similarly, lycopene production from MurNAc by *C. glutamicum* $\Delta crtYEb\Delta nanR$  *PCQ* was comparable to that observed by similar strains with 100 mM glucose [ $30 \pm 10 \mu\text{g}\cdot\text{g}(\text{CDW})^{-1}$ ] and 100 mM GlcNAc [ $29.6 \pm 4.5 \mu\text{g}\cdot\text{g}(\text{CDW})^{-1}$ ] (Heider et al., 2012; Matano et al., 2014). To establish viable production processes with MurNAc as sole or combined carbon source, more work to increase titres, yields and volumetric productivities is needed. Conceptually, however, this work laid the foundation for recycling the cell wall fraction of bacterial biomass from large-scale production processes as substrate for fermentative production of food and feed additives.

## CONCLUSION

*Corynebacterium glutamicum* was successfully metabolically engineered for utilization of the amino sugar MurNAc as alternative carbon source for growth and production of relevant value-added compounds, specifically L-lysine, L-glutamate and lycopene, from this carbon source lacking competing uses in human and animal nutrition.

## AUTHOR CONTRIBUTIONS

VW conceived the study. VW and ES planned the experiments. ES and LB performed and analyzed the experiments. ES and LB drafted the manuscript. VW finalized the manuscript. All the authors agreed to the final version of the manuscript.

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# A Screening Method for the Isolation of Bacteria Capable of Degrading Toxic Steroidal Glycoalkaloids Present in Potato

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Potato juice, a by-product of starch processing, is a potential high-value food ingredient due to its high protein content. However, conversion from feed to human protein requires the removal of the toxic antinutritional glycoalkaloids (GAs)  $\alpha$ -chaconine and  $\alpha$ -solanine. Detoxification by enzymatic removal could potentially provide an effective and environmentally friendly process for potato-derived food protein production. While degradation of GAs by microorganisms has been documented, there exists limited knowledge on the enzymes involved and in particular how bacteria degrade and metabolize GAs. Here we describe a series of methods for the isolation, screening, and selection of GA-degrading bacteria. Bacterial cultures from soils surrounding greened potatoes, including the potato peels, were established and select bacterial isolates were studied. Screening of bacterial crude extracts for the ability to hydrolyze GAs was performed using a combination of thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and liquid chromatography mass spectrometry (LC-MS). Analysis of the 16S rRNA sequences revealed that bacteria within the genus *Arthrobacter* were among the most efficient GA-degrading strains.

**Keywords:** glycoalkaloids,  $\alpha$ -chaconine,  $\alpha$ -solanine, potato fruit juice, soil bacteria, *Arthrobacter*, microbial enzymes

## INTRODUCTION

Globally, plant-produced starch is a dominant nutrient source for millions of people, and among the most important starch-producing plants are potatoes (Ellis et al., 1999; Burrell, 2003; Jobling, 2004; Sonnewald and Kossmann, 2012). In addition to serving as a major nutrient source, potato starch is also an important industrial food ingredient. A by-product of the potato starch industry is potato fruit juice (PFJ) which contains proteins, amino acids and peptides (Zhang et al., 2017). While PFJ has a high nutritional value, vegetable proteins, including potato, contain many anti-nutritional components and thus must be refined prior to human consumption (Steinhof et al., 2005). In the case of potato proteins, the value is reduced due to the presence of toxic steroidal glycoalkaloids (GAs), polyphenols and the enzyme polyphenol oxidase (PPO) responsible for discoloration causing the final products to have a brown hue and consequently a negative affect on both solubility

and digestibility (Prigent et al., 2007). GAs form part of the potato plants' natural defense and have been shown to have toxic effects in humans causing among others gastrointestinal and neurological disorders (e.g., vomiting, diarrhea, headache, and hallucinations) (Grunenfelder et al., 2006). In addition, GAs add an undesirable bitter taste to potato protein-based products (Sinden et al., 1976). Consequently, these components must be removed from the potato protein fraction before it can be suitable for human consumption. Enzymatic removal of the carbohydrate moiety could provide a more sustainable and environmentally friendly process for detoxifying PFJ. A recent study by Koffi et al. (2017) demonstrated that a chemoenzymatic treatment of GAs using partial acidic hydrolysis combined with an enzymatic treatment enabled the efficient conversion of  $\alpha$ -chaconine to solanidine. Solanidine is also a valuable product which can serve as a precursor for hormone synthesis and other pharmacologically active compounds (Vronen et al., 2003; Koffi et al., 2017). The enzyme used in the study was a  $\beta$ -glycosidase from the cockroach *Periplaneta americana* (Koffi et al., 2017). To that end, we wanted to identify bacterial isolates capable of producing specific enzymes capable of degrading toxic potato-derived GAs into solanidine.

It has previously been established that both potato-derived extracts and extracts of fungal derived pathogens contain GA-degrading activities (Weltring et al., 1997; Oda et al., 2002; Nikolic et al., 2005; Dahlin et al., 2017). Some of these enzymes appear to act by sequential degradation of the carbohydrate moiety, indicating that multiple enzymes may be required for complete deglycosylation of the alkaloid. Furthermore, the enzymes that can degrade alkaloids may be specific for only one of the two main GAs produced in potato,  $\alpha$ -chaconine and  $\alpha$ -solanine. These compounds are found in all parts of the plant and are typically produced by plants as biopesticides, or when the plant is damaged. Potato GAs consist of the solanidine aglycone with a carbohydrate side-chain, which is important for mediating interactions with cell membranes (Koffi et al., 2017). It has been proposed that in order to detoxify  $\alpha$ -chaconine and  $\alpha$ -solanine, the first step is removal of the trisaccharide to release solanidine. However, a recent study showed solanidine to have a great impact on the physiology of the phytopathogen *Phytophthora infestans* compared to its glycosylated form (Dahlin et al., 2017). Only scarce information and few papers currently describe the microbes and enzymes involved in the degradation of potato GAs and therefore new knowledge is urgently needed. To begin to fill this information gap we here describe a series of methods for the identification of GA-degrading bacteria.

In this study, we aimed to develop a pipeline for the isolation, screening and selection of bacteria capable of degrading  $\alpha$ -chaconine and  $\alpha$ -solanine. Enrichment cultures were established to isolate bacterial strains capable of tolerating high concentrations of GAs or of utilizing them as a sole carbon source. Using a combination of low-tech [enrichment cultures, thin layer chromatography (TLC)] and high-tech [liquid chromatography mass spectrometry (LC-MS)] techniques we successfully isolated and screened bacterial strains capable of degrading GAs demonstrating the utility of this pipeline as a starting point for enzyme discovery. To the best of our

knowledge, this is the first report describing bacteria capable of degrading potato GAs.

## MATERIALS AND METHODS

### Extraction of $\alpha$ -Chaconine and $\alpha$ -Solanine and Preparation of Standards

Crude extraction of (GAs) from potato protein supplied by Kartoffelcentralen (KMC), Amba was performed as follows: 0.5 g of potato protein was added to 5 ml methanol-water-acetic acid (at a ratio of 80:9.5:0.5) in a 50 ml tube and shaken vigorously for 10 min. The tube was centrifuged 10 min at 4°C and the upper phase gently decanted into a fresh 50 ml tube. Extractions were pooled and dried in a vacuum centrifuge before resuspension in 1.5 ml filter-sterilized potassium phosphate buffer at pH 7.5. To improve the solubility of the GAs, 1.5  $\mu$ l acetic acid was added. The crude extract was filter-sterilized using a 0.45  $\mu$ m filter after which the purified GAs were detected and identified by HPLC analysis (see below).

Pure  $\alpha$ -chaconine was obtained from PhytoLabs (Vestenbergsgreuth, Germany) and  $\alpha$ -solanine was purchased from either PhytoLabs or Sigma-Aldrich (Brøndby, Denmark).

### Isolation and Purification of Bacterial Strains From Potato Soil Extracts

Bacteria were isolated on potato dextrose agar at fifth strength (PDA 1/5) by inoculation of material from greened potatoes and the surrounding soil. Alternatively, minimal media as described by Nan and Edens, 1998 was supplemented with a pure or crude extraction of GAs (**Supplementary Figure S1**) was used for isolation of bacteria. Plates were incubated at 15°C for a week and bacterial colonies were isolated for purification. Following three rounds of isolation to obtain pure cultures, selected strains were routinely cultured in either Lysogeny broth (LB) or on LB agar at 20°C for 1–2 days, depending on the strain.

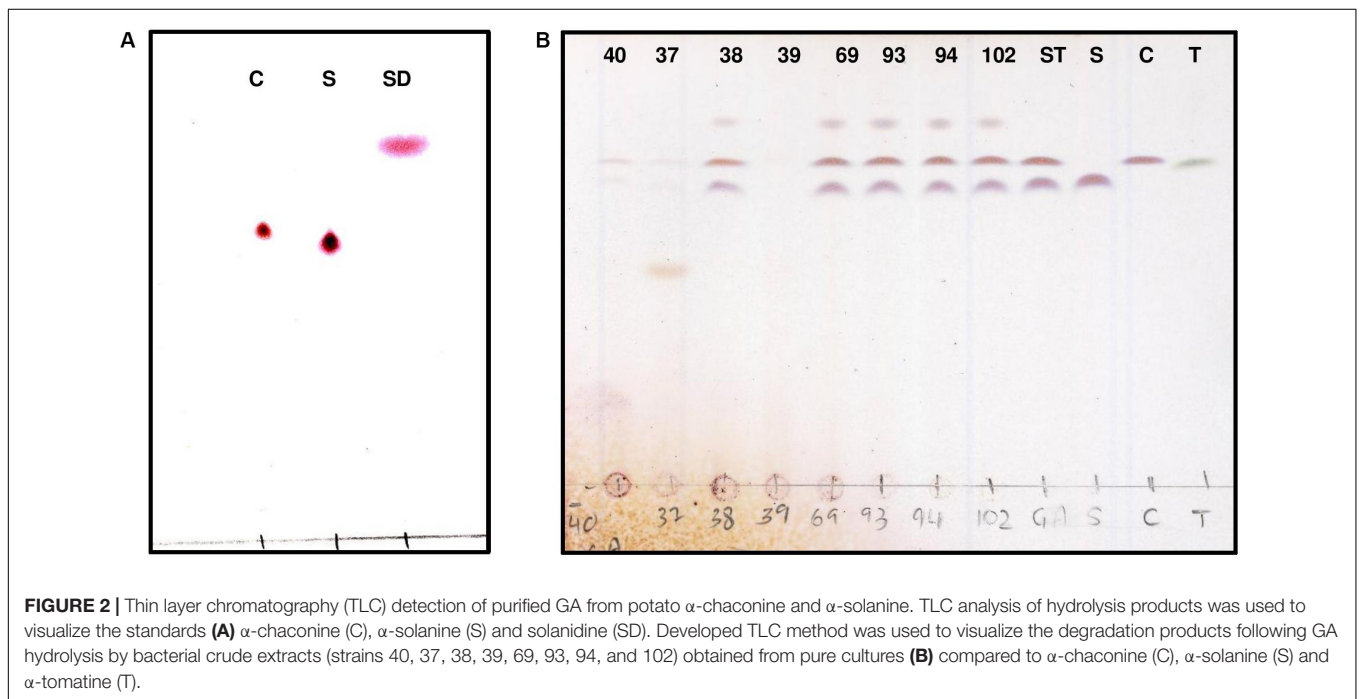
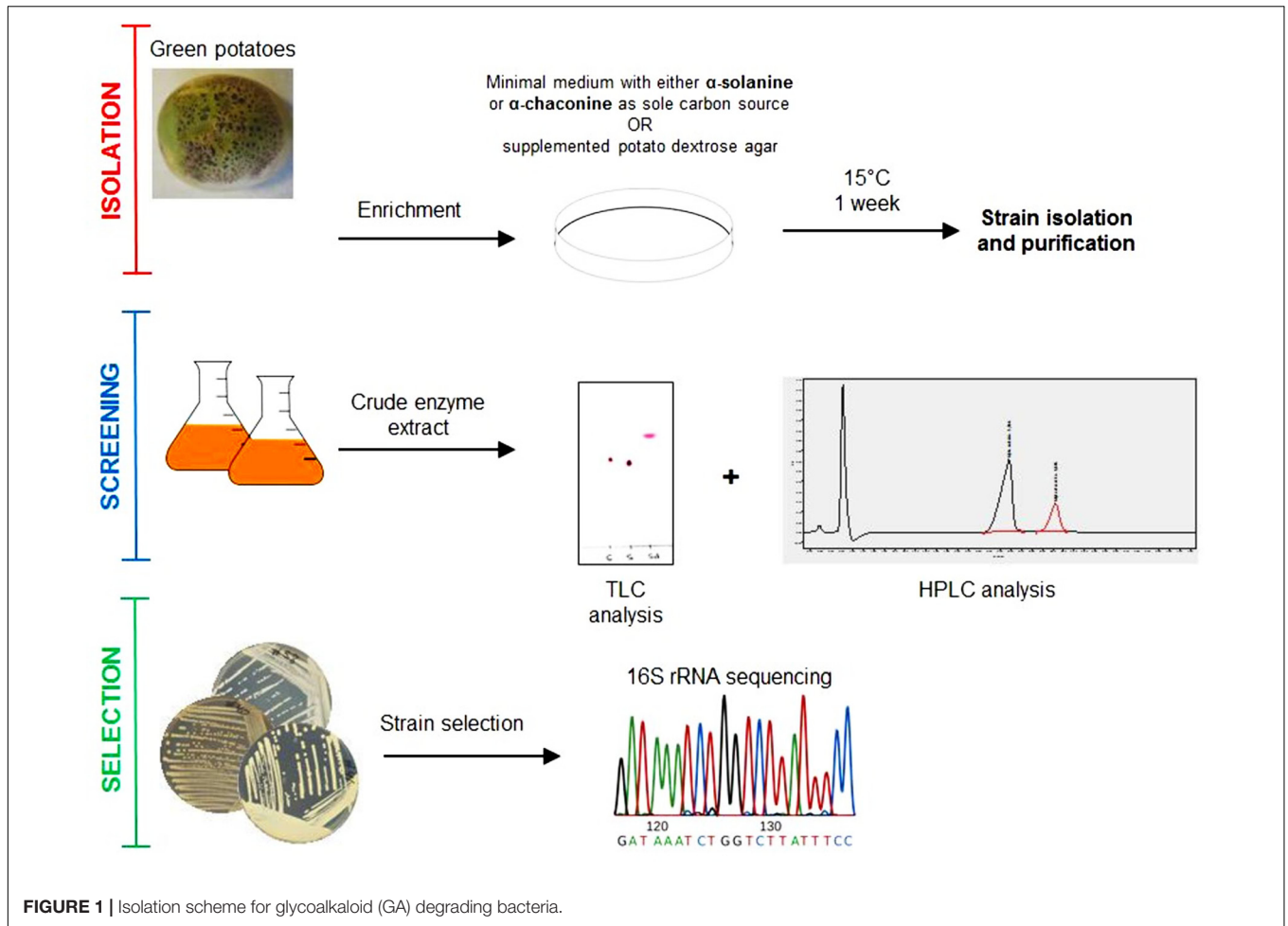
### Screening for Bacteria Capable of Using $\alpha$ -Chaconine or $\alpha$ -Solanine as a Sole Carbon Source

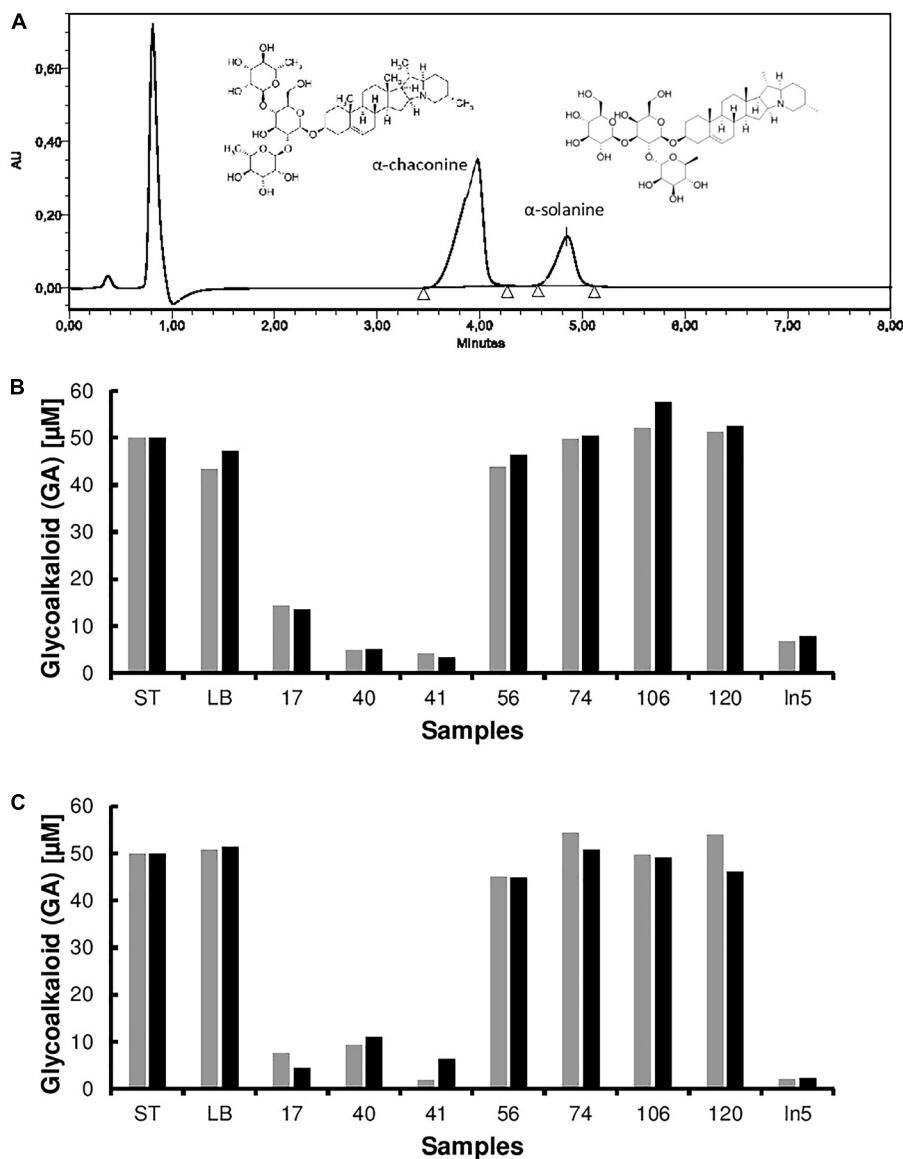
To determine whether the bacterial isolates could use the GAs as a sole carbon source, the strains were grown overnight in LB at 20°C and 5  $\mu$ l culture plated on Petri dishes containing minimal media as described above enriched with either  $\alpha$ -chaconine or  $\alpha$ -solanine as a sole carbon source. Strains that scored positive for growth were selected for further characterization.

### DNA Extraction and Determination of 16S Ribosomal RNA Sequences

Extraction of crude DNA from the isolated bacterial cultures was performed as follows. Bacterial cells were scraped off a Petri dish and resuspended in 1 ml sterile phosphate-buffered saline (1  $\times$  PBS) buffer in a sterile Eppendorf tube. The cells







**FIGURE 3 |** High performance liquid chromatography (HPLC) spectra of the pure glycoalkaloids (GA)  $\alpha$ -chaconine and  $\alpha$ -solanine from potato. HPLC analysis of hydrolysis products was used to quantify GA degradation by bacterial extracts from pure cultures (A). Degradation of potato-derived glycoalkaloids  $\alpha$ -chaconine (black) and  $\alpha$ -solanine (gray) by crude extracts obtained from bacterial cell cultures (strains 17, 40, 41, 56, 74, 106, 120, and In5) compared to the control (media blank LB) and standard (ST) induced (B) or non-induced (C).

were concentrated by centrifugation for 5 min at  $10,000 \times g$  after which the supernatant removed. For extraction of DNA, the pellet was resuspended in 100  $\mu$ l sterile water and boiled 10 min and immediately placed on ice before adding 900  $\mu$ l ice-cold sterile water. Cell debris was pelleted by centrifugation for 5 min at  $10,000 \times g$ . The supernatant containing DNA was transferred to a clean tube ready for PCR analysis or storage at  $-20^{\circ}\text{C}$ . The 16S rRNA gene sequence was determined by PCR using the universal bacterial 16S rRNA primers: 16S-27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S-1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') (Webster et al., 2006). Sanger sequencing of the 16S rRNA gene was performed

by GATC-Biotech, Konstanz, Germany, for the identification of select strains.

## Enzyme Assays

For preliminary screening, strains were grown overnight in 5 ml LB shaking at 200 rpm at  $20^{\circ}\text{C}$  and then diluted 1:100 into LB medium either water (control) or  $10 \mu\text{g ml}^{-1}$  final concentration of  $\alpha$ -chaconine or  $\alpha$ -solanine and incubated 20 h under the same conditions. To obtain crude extracellular extracts, cultures were centrifuged at  $20^{\circ}\text{C}$  for 30 min at  $4000 \times g$  and either used directly or frozen at  $-20^{\circ}\text{C}$ . Enzyme reactions consisting of 10  $\mu$ l 50 mM sodium acetate buffer pH 5.5, 40  $\mu$ l substrate

**TABLE 1** | 16S ribosomal RNA sequences of isolates.

Isolate	Closest relative	Genbank accession	Identity scores (%)
37	<i>Arthrobacter</i> sp. PJ8	MH245050	99
39	<i>Arthrobacter</i> sp. X49	MH245051	99
40	<i>Serratia</i> sp. Y4	MH245052	98
41	<i>Arthrobacter</i> sp. PJ8	MH245053	99

(0.2 mM GA in 100 mM sodium acetate buffer pH 5.0) and 45  $\mu$ l of crude extract was set up and incubated 24 h at 20°C. Enzyme reactions were terminated by heating for 10 min at 90°C. After centrifugation or 10 min at 17,000  $\times$  g, the supernatant was transferred to a fresh 1.5 ml tube and dried in a vacuum for further analysis. Pellets were resuspended in 20  $\mu$ l of methanol and 8  $\mu$ l were spotted on TLC plates for analysis or 10  $\mu$ l were characterized by HPLC analysis.

For selecting strains for further characterization, strains were grown overnight in 5 ml LB shaking 200 rpm 20°C and then washed with 0.9% NaCl and standardized to an OD<sub>600 nm</sub> of 1.0. To 100 ml shake flask, 1 ml of bacterial culture (final OD<sub>600nm</sub> of 0.1) was added to 9 ml LB supplemented with acidic phosphate buffer (control) or a glycoalkaloid mix (5  $\mu$ g ml<sup>-1</sup> each of  $\alpha$ -chaconine,  $\alpha$ -solanine, and  $\alpha$ -tomatine) and grown shaking 200 rpm 20°C for 20 h. To obtain crude extracellular extracts, cultures were centrifuged at 20°C for 20 min at 4000  $\times$  g and used directly for enzyme activity analysis. Enzyme assays consisting of 10  $\mu$ l 50 mM sodium acetate buffer pH 5.5, 40  $\mu$ l substrate (0.2 mM of  $\alpha$ -chaconine and 0.2 mM of  $\alpha$ -solanine) in 100 mM sodium acetate buffer pH 5.0 and 45  $\mu$ l of crude extract were set up and incubated at 20°C for 35 h. Enzyme reactions were terminated by heating for 10 min at 90°C and subsequently centrifuged for 10 min at 17,000  $\times$  g before transferring the supernatant to either an HPLC vial (60  $\mu$ l) for semi-quantitative analysis or to a fresh 1.5 ml tube (30  $\mu$ l) for qualitative TLC analysis. For TLC analysis, 30  $\mu$ l extract was dried 1 h in a vacuum centrifuge and resuspended in 7  $\mu$ l cold methanol. Samples were briefly centrifuged and 5  $\mu$ l spotted on TLC plate. TLC plates were run twice in *n*-butanol-acetic acid-water (2:1:1) solvent system and sprayed with 50% vv<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> and heated for 10 min at 85°C. For HPLC analysis, a 25  $\mu$ l sample was injected and conditions for analysis were as described below. For analysis of intermediate metabolites produced during degradation or detection of solanidine, 30  $\mu$ l of sample was analyzed by LC-MS.

### Thin Layer Chromatography (TLC)

TLC was performed on silica gels 60 F254 (Merck) using *n*-butanol-acetic acid-water (2:1:1) as a running solvent and plates were developed on a hot plate after spraying with 50% vv<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>.

### High Performance Liquid Chromatography (HPLC) Analysis

Quantification and identification of  $\alpha$ -chaconine and  $\alpha$ -solanine were performed using a Waters 2695 Alliance system with Waters 2487 Dual absorbance detector at 202 nm. The column was a Waters Nova-Pak C<sub>18</sub> with 4  $\mu$ m particle size, operated at 40°C.

Solvent were 60% acetonitrile and 40% phosphate buffer at pH 7.6 (mixture of K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> to obtain the correct pH value) at a flow rate of 1.5 ml/min.

### Liquid Chromatography Mass Spectrometry (LC-MS) Analysis

Quantification and identification of  $\alpha$ -chaconine and  $\alpha$ -solanine was based on the ion intensity of the single charged monoisotopic mass ( $\alpha$ -chaconine, m/z 852.51;  $\alpha$ -solanine, m/z 868.51). The presence of solanidine was monitored at m/z 398.34. Ion intensities were normalized to the level in untreated LB media. Samples were analyzed in an LC-MS setup with an Eksigent nanoLC 415 system (Sciex) connected to a TripleTOF 6600 mass spectrometer (Sciex) equipped with a NanoSpray III source (AB Sciex). Ion-intensities were extracted in Peakview 1.2 (Sciex).

## RESULTS AND DISCUSSION

A previous study demonstrated a rapid turnover of potato (GAs) in top soils of Danish potato fields, presumably due to microbial metabolism (Jensen et al., 2009). Therefore, it is likely that bacteria producing GA-degrading enzymes are present in such environments. In addition, green potato peel is known to contain significantly higher amounts of toxic GAs (Omayio et al., 2016). Based on these observations, a screening strategy for GA-degrading bacteria was established as shown in **Figure 1**. Potato extracts from green potato peel and the immediate surrounding soil were used as a potential source of GA-degrading bacteria. To select for such bacteria, a series of enrichment cultures were established using commercially purified GAs or a crude extract produced from potato powder (**Supplementary Figure S1**). A crude preparation of GAs was made as pure  $\alpha$ -chaconine and  $\alpha$ -solanine are expensive products. Hence, when using pure compounds small petri dishes with a small volume of media were used. From these enrichment cultures a total of 85 isolates were obtained and used for further analysis. To determine whether the isolated strains could utilize GAs as a sole carbon source, cultures grown overnight were transferred to petri dishes with minimal medium and supplemented with either  $\alpha$ -chaconine or  $\alpha$ -solanine as a sole carbon source. A total of 14 strains that grew on the GA-enriched media were transferred to liquid media containing either a mixture of GAs or no GAs and cultured overnight. Crude bacterial extracts were screened for GA degradation using an optimized and rapid TLC method for the detection of both  $\alpha$ -chaconine or  $\alpha$ -solanine (**Figure 2** and **Supplementary Figure S2**). In addition to these two compounds, the aglycone solanidine that is produced upon removal of the carbohydrate moiety of the two compounds, was included in the TLC analysis (**Figure 2**). Parallel to the TLC analysis, GA degradation was also confirmed by HPLC and LC-MS analysis, respectively (**Figure 3** and **Supplementary Figures S3, S4**). Interestingly, no solanidine was detected in samples where bacterial extracts were incubated with GAs, indicating that the isolates may be capable of the complete hydrolysis and metabolism of GAs and their degradation products. The only GA related intermediate product detected by LC-MS was

a 705.5 Da GA variant corresponding to either  $\alpha$ -chaconine or  $\alpha$ -solanine were the outermost saccharide have been removed by hydrolysis resulting in solanidine modified with a disaccharide (**Supplementary Figure S4**).

Of the 14 GA degrading bacterial strains isolated from a series of enrichment cultures, four strains S37, S39, S40, and S41, were the best at degrading potato GAs. Unsurprisingly, the four strains were all isolated from minimal media solidified with agar containing a mixture of pure  $\alpha$ -chaconine and  $\alpha$ -solanine as a sole carbon source. Enrichment cultures containing GAs as a sole carbon source was the best method to enable the isolation of GA degrading bacteria.

While the microbial degradation of GAs is known, few studies report bacteria capable of degrading GAs. For example, *Clavibacter michiganensis* and *Streptomyces scabies* are bacteria that can degrade the tomato GA  $\alpha$ -tomatine, however, to the best of our knowledge no studies report the bacterial degradation of potato GAs (Kaup et al., 2005; Seipke and Loria, 2008). During screening of the strain collection, we observed that one strain, *Serratia* sp. S40 also displayed antifungal activity. In order to investigate whether other biocontrol bacteria can degrade GAs, we tested the ability of the well-described antifungal strain *Pseudomonas fluorescens* In5 isolated from a potato field in southern Greenland (Michelsen and Stougaard, 2011; Hennessy et al., 2017). We observed that *P. fluorescens* In5 was able to degrade both  $\alpha$ -chaconine and  $\alpha$ -solanine and similar to the GA-degraders described in this study, appeared to be capable of the complete degradation and metabolism of both compounds as no solanidine was detected following hydrolysis of the GAs by a crude enzyme extract from *P. fluorescens* In5. Further studies investigating the mechanisms by which these bacteria degrade GAs will be required.

Identity of the four strains that produced GA-degrading extracts was done by analysis of 16S rRNA gene sequences (**Table 1**). Three isolates (S37, S39, and S41) showed more than 99% sequence identity to species of the genus *Arthrobacter* and the fourth isolate (S40) showed 98% sequence identity to species of the genus *Serratia*. All strains were capable of degrading both  $\alpha$ -chaconine and  $\alpha$ -solanine. Published information on GA-degrading bacteria is sparse, but it has previously been reported that an  $\alpha$ -chaconinase enzyme produced by *Gibberella pulicaris* is induced by  $\alpha$ -chaconine,  $\alpha$ -solanine and the tomato glycoalkaloid  $\alpha$ -tomatine (Becker and Weltring, 1998). All four strains were able to degrade  $\alpha$ -chaconine and  $\alpha$ -solanine independent from whether bacterial cultures were induced by the addition of GAs. Further characterization of the four selected strains is now

necessary to determine their species and elucidate the enzymes involved in GA degradation.

The isolation method here described identified four strains, which demonstrated the ability to metabolize and degrade the potato-derived GAs  $\alpha$ -chaconine and  $\alpha$ -solanine. The 16S rRNA gene sequences of these strains showed highest similarity to strains belonging to genera *Arthrobacter* and *Serratia*. Functional genomics of the identified strains is now required to identify the genes and metabolic pathways related to the biodegradation of potato GAs.

## CONCLUSION

We successfully developed a screening method to isolate GA-degrading bacteria from potato soil extracts. This will enable the future identification and characterization of enzymes involved in the degradation of GAs to upgrade industrial waste products into valuable food ingredients.

## AUTHOR CONTRIBUTIONS

RH and PS conceived the study. RH, NJ, and CS performed the experiments. All authors analyzed and interpreted the data, drafted and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.02648/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Diversifying of Chemical Structure of Native *Monascus* Pigments

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Red Yeast Rice, produced by solid state fermentation of *Monascus* species on rice, is a traditional food additive and traditional Chinese medicine. With the introduction of modern microbiology and biotechnology to the traditional edible filamentous fungi *Monascus* species, it has been revealed that the production of red colorant by fermentation of *Monascus* species involves the biosynthesis of orange *Monascus* pigments and further chemical modification of orange *Monascus* pigments into the corresponding derivatives with various amine residues. Further study indicates that non-*Monascus* species also produce *Monascus* pigments as well as *Monascus*-like pigments. Based on the chemical modification of orange *Monascus* pigments, the diversification of native *Monascus* pigments, including commercial food additives of Red *Monascus* Pigments<sup>®</sup> and Yellow *Monascus* Pigments<sup>®</sup> in Chinese market, was reviewed. Furthermore, *Monascus* pigments as well as their derivatives as enzyme inhibitors for anti-obesity, hyperlipidemia, and hyperglycemia was also summarized.

**Keywords:** Red Yeast Rice, *Monascus* pigments, non-*Monascus* species, chemical modification, enzyme inhibitor

## INTRODUCTION

Filamentous fungi, known as a prolific producer of secondary metabolites, are an important resource for discovering small molecules of pharmaceutical (such as penicillin, lovastatin, cyclosporine, etc.) as well as food colorant additives (Dufossé et al., 2014). One of the famous examples is the production of Red Yeast Rice (a traditional Chinese food colorant and medicine) by *Monascus* species (Ma et al., 2000). *Monascus* species were first screened in Red Yeast Rice and characterized in 1884 (van Tieghem, 1884). After then, modern microbiology and biotechnology are introduced to the traditional edible filamentous fungi. Many secondary metabolites of *Monascus* species are isolated and identified, such as monacolin K (Endo, 1980), citrinin (Blanc et al., 1995), and *Monascus* pigments. At the same time, the negative and positive bioactivity of the secondary metabolites are also recognized. There are many comprehensive reviews about the molecular biology of *Monascus*, the secondary metabolites as well as their metabolite bioactivity (Juzlova et al., 1996; Feng et al., 2012; Chen et al., 2015).

Orange *Monascus* pigments, including rubropunctatin (1) and monascorubrin (2) with a classic azaphilone structure (Figure 1), are key color components of *Monascus* fermentation. In the present work, we focus on the structure diversity of orange *Monascus* pigment derivatives as well as their corresponding characters and bioactivities. First, progress on the biosynthetic pathway of orange *Monascus* pigments was updated. Then, production of orange *Monascus* pigments as well as *Monascus*-like pigments by non-*Monascus* species was introduced. And then, the diversification

of *Monascus* pigment structure, including commercial food additives of Red *Monascus* Pigments® and Yellow *Monascus* Pigments® in Chinese market, by chemical modification of orange *Monascus* pigments as well as their derivatives as enzyme inhibitors for anti-obesity, hyperlipidemia, and hyperglycemia was also summarized.

## BIOSYNTHESIS OF *Monascus* PIGMENTS

*Monascus* pigments, such as rubropunctatin (**1**) and monascorubrin (**2**), are chemicals by the esterification of azaphilone with  $\beta$ -ketoacid. The biosynthesis of  $\beta$ -ketoacid is catalyzed by fatty acid synthase (FAS) while azaphilone is catalyzed by polyketide synthase (PKS). Both polyketides and fatty acids are architected by iteratively decarboxylative Claisen thioester condensations of an activated acyl starter unit with malonyl-CoA-derived extender units (Smith and Tsai, 2007). The difference is that FAS typically catalyzes a full reductive chain after each elongation while PKS catalyzes an optional reductive cycle that can be partly or fully omitted before the next round of elongation (Hertweck, 2009). Nuclear magnetic resonance analysis after feeding with  $^{13}\text{C}$ -labeled acetate during submerged cultures is applied to investigate the biosynthetic pathway of *Monascus* pigments. Results indicate that fatty acid biosynthesis and polyketide biosynthesis shares acetate and propionate as the simplest biosynthetic building blocks (Hajjaj et al., 2000). Furthermore, it is also confirmed that tetraketide is the precursor of *Monascus* pigments in the biosynthetic pathway (Hajjaj et al., 1999). Recently, the biosynthetic pathway of *Monascus* pigments becomes more and more clear with the progress of modern molecular biology (Balakrishnan et al., 2013; Chen et al., 2017).

### Biosynthetic Pathway of *Monascus* Pigments

Gene cultures for biosynthesis of *Monascus* pigments had been studied extensively by two groups, i.e., Chen's group of Huazhong Agricultural University (China) (Chen et al., 2017) and Kwon's group of Myongji University (South Korea) (Balakrishnan et al., 2013). Based on the gene cultures from various *Monascus* species, some highly conserved and nearly identical genes in all *Monascus* species are summarized (**Figure 2A**). Those genes are divided into two regions, i.e., region I indicating as filled arrows and region II as open arrows, which encode the core enzymes for *Monascus* pigment biosynthesis. Between region I and region II, there are some genes for special *Monascus* species, which is shown as light blue box in **Figure 2A**. For examples, there is an ankyrin repeat protein-encoding gene in *M. ruber* M7 and two *M. purpureus* strains. *M. ruber* NRRL 1597 and *M. pilosus* contain six genes related to transport and signal transduction (Chen et al., 2017).

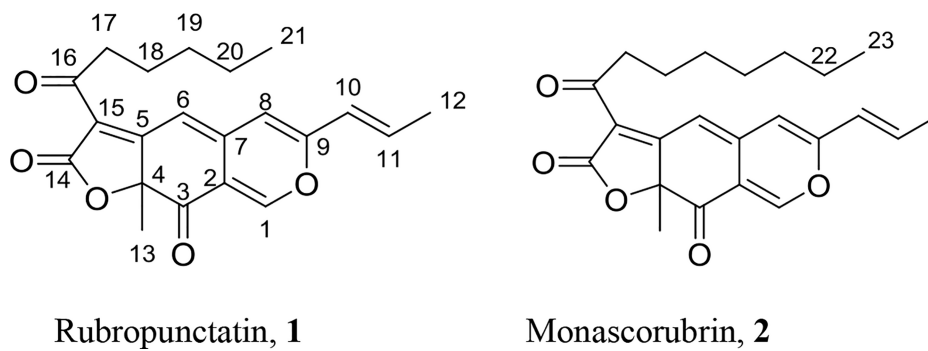
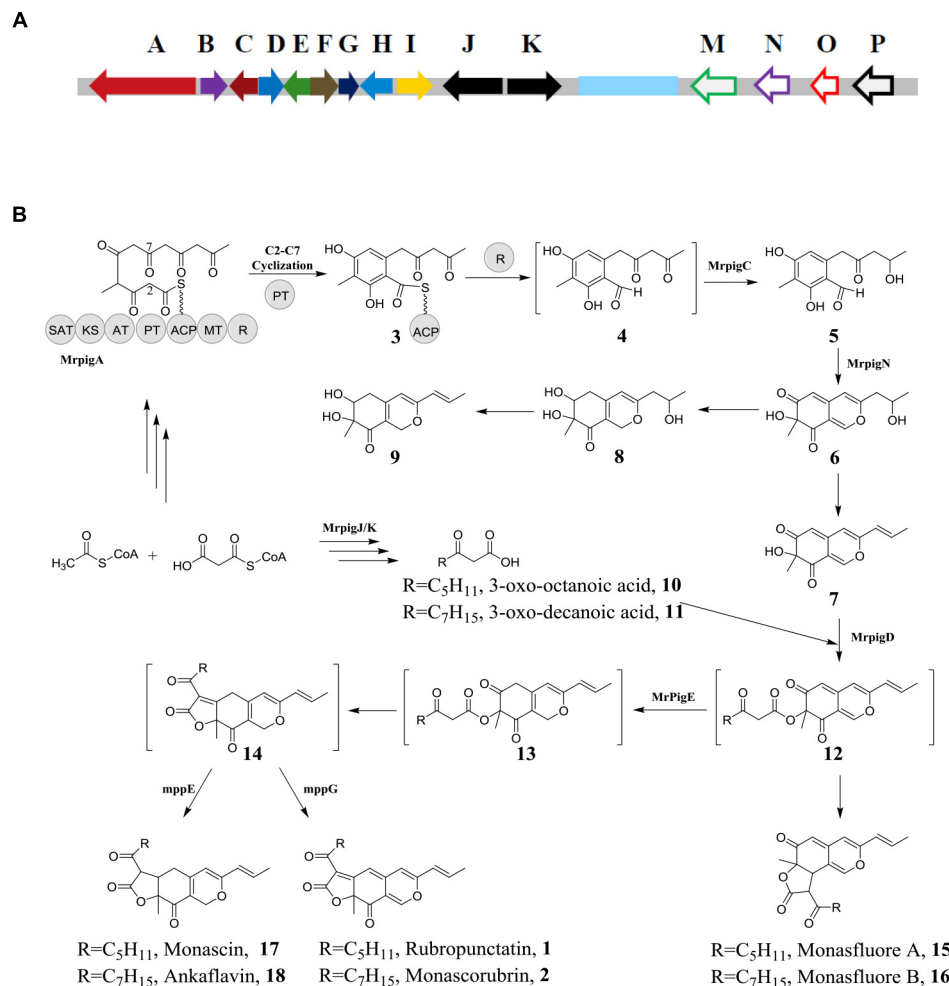
Among the gene clusters of region I, Mrpig A gene, corresponding to MpPKS5 gene of *Monascus purpureus* (Balakrishnan et al., 2013), encodes PKS and catalyzes biosynthesis of the key aromatic ring intermediate (**4**)

(**Figure 2B**). Similarly, Mrpig N gene in region II, corresponding to mpp 7 gene of *Monascus purpureus* (Balakrishnan et al., 2014a), encodes a FAD-dependent monooxygenase and catalyzes the hydroxylation reaction at the C-4 carbon of compound **5**. Thus, the key azaphilone structure chemical **6** can be biosynthesized. Many filamentous fungi are sources of secondary metabolites with bicyclic azaphilone structures. Similar gene clusters are also identified, such as *Aspergillus niger* (Zabala et al., 2012). Different from other filamentous fungi, there is Mrpig E gene in region II of *Monascus* gene clusters, corresponding to mpp C gene of *Monascus purpureus* (Balakrishnan et al., 2014b), encodes NAD(P)H-dependent oxidoreductase and catalyzes the reduction of compound **12** into **13**. The chemical **13** is the key to formation of the tricyclic ring of *Monascus* pigments. The pathway for biosynthesis of *Monascus* pigments is detailed below.

### Biosynthesis of Azaphilone Structure

Fungal PKSs can be classified into three groups according to the function and phylogeny, i.e., non-reducing PKSs, partial-reducing PKSs, and highly reducing PKSs (Chooi and Tang, 2012). The biosynthesis of *Monascus* pigments starts with the assembly of a hexaketide backbone catalyzed by the non-reducing PKS encoded by Mrpig A gene. As shown in **Figure 2B**, the PKS includes several units, such as the starter acyl transferase (SAT) domain selects an acetyl-CoA starter unit, and the ketoacyl synthase (KS)-acyl transferase (AT)-acyl carrier protein (ACP) domains extend this starter unit five times with malonyl-CoA in five successive decarboxylative Claisen condensation cycles. The methyltransferase (MT) domain conducts a single C-methylation at C-4 carbon. The reactive hexaketide chain then undergoes a product template (PT) domain-mediated C-2 to C-7 aldol cyclization to afford the first aromatic ring chemical **3** (Li et al., 2010; Xu et al., 2013; Liu et al., 2015). The first ring chemical **3** follows reductive release chemical **4** catalyzed by reductive releasedomain (R) (Bailey et al., 2007). Mrpig C catalyzes the transformation of chemical **4** to **5** via  $\omega$ -1 carbonyl to the alcohol. The chemical **5** has been isolated and identified from the broth of *Monascus ruber* mutant (Liu et al., 2016).

Mrpig N is critical to morphing the chemical **5** into the bicyclic pyran-containing azaphilone core, which is confirmed by *in vivo* experiment via knockout of Mrpig N (Chen et al., 2017). Hydroxylation at C-4 carbon of chemical **5** is catalyzed by a FAD-dependent monooxygenase encoded by Mrpig N, which leads to de-aromatization of the ring and then induce keto-enol tautomerization at the C-1 aldehyde. Finally, the condensation between the C-1 enol and the C-9 carbonyl to afford the pyran ring, i.e., the key azaphilone chemical **6**. Chemical **7** (Woo et al., 2014) and chemical **8/9** (Jongrungruangchok et al., 2004) are also isolated and identified, which are resulted from the further modification of the key azaphilone chemical **6** by enzymatic or non-enzymatic reaction. Fatty acid 3-oxo-octanoic acid (**10**) and 3-oxo-decanoic acid (**11**) are produced catalyzed by FAS (Balakrishnan et al., 2014c; Liang et al., 2018). Mrpig D encodes acyltransferase, which catalyzes the transferring of the fatty acids (**10/11**) to the C-4 alcohol of chemical **7** and formation of the anticipated intermediate **12**.

**FIGURE 1** | Chemical structure of orange *Monascus* pigments.**FIGURE 2** | Biosynthesis of orange *Monascus* pigments. **(A)** Conserved genes involving in biosynthesis of *Monascus* pigments; **(B)** biosynthetic pathway of *Monascus* pigments.

## Formation of the Tricyclic Ring of *Monascus* Pigments

Different from most of azaphilones, the tricyclic ring of *Monascus* pigments is established by intramolecular Knoevenagel aldol

condensations of key intermediate **12**. Mrpig E, an ortholog of mpp C (98% identity), determines the region-selectivity of the spontaneous Knoevenagel condensation. It is proposed that the NAD(P)H-dependent oxidoreductase encoded by Mrpig E

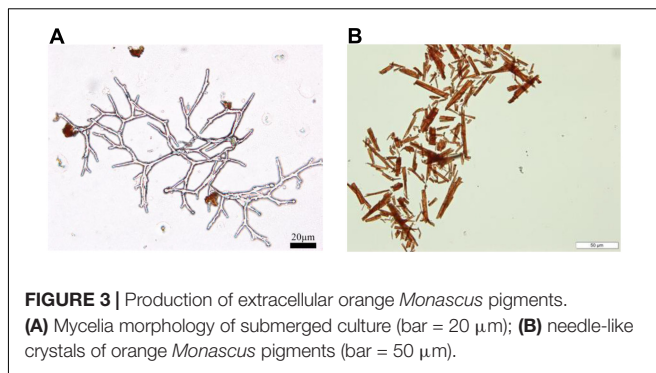


gene catalyzes the reduction of compound **12** into a chemical **13**. This reductive reaction eliminates the  $\pi$ -conjugated system in chemical **12** and makes C-5 carbon of chemical **13** a better electron acceptor. Thus, the anticipated linear tricyclic intermediate **14** is produced by Knoevenagel condensation. A  $\Delta$ mpp C mutant produces monasfluore A/B (**15/16**) by Knoevenagel condensation between  $\alpha$  carbon in the 3-oxo-fatty acyl moiety and the C-3 carbonyl group of chemical **12** (Balakrishnan et al., 2014b). In general, majority of chemicals **12** are channeled by Mpig E through the main pathway toward the linear tricyclic intermediate **14** and the angular chemicals **15/16** are also yielded at a low level (Huang et al., 2008).

An oxidase, encoded by mpp G gene (corresponding to Mrpig F in **Figure 2A**), controls transformation of intermediate **14** into orange *Monascus* pigments (**1/2**). A  $\Delta$ mpp G mutant abolishes the biosynthesis of orange *Monascus* pigments while no significant alteration the level of yellow *Monascus* pigments (**17/18**). This result indicates that yellow *Monascus* pigments can be biosynthesized independently from orange *Monascus* pigments. On the other hand, feeding of yellow *Monascus* pigment (**17**) to  $\Delta$ MpPKS5 mutant also excludes the possibility of conversion of yellow *Monascus* pigment into the corresponding orange one (**1**) (Balakrishnan et al., 2017b). Similarly, a reductase, encoded by mpp E gene (corresponding to Mrpig H in **Figure 2A**), controls the conversion of the intermediate **14** into yellow *Monascus* pigments (**17/18**) (Balakrishnan et al., 2017a). The key role of intermediate **14** to the biosynthesis of *Monascus* pigments is also confirmed via analysis of Mrpig E by gene disruption, complementation and overexpression in *Monascus ruber* (Liu et al., 2014). The results demonstrate that the Mrpig E deletion strain fails to produce orange *Monascus* pigments and the Mrpig E complementation strain recovers the ability to production of orange *Monascus* pigments. However, there are still detectable yellow *Monascus* pigments in the broth after culture of  $\Delta$ mpp E mutant (Balakrishnan et al., 2017a). Further experiment indicates the  $\Delta$ mpp DEG (mpp D corresponding to Mrpig G in **Figure 2A**) mutant remains the ability for production of orange *Monascus* pigments as well as yellow ones (Balakrishnan et al., 2017a). These results hint that multiple discrete routes may be involved in the biosynthesis of *Monascus* pigments.

## Production of Extracellular Orange *Monascus* Pigments

As shown in **Figure 2A**, there is Mrpig P gene in the gene clusters for biosynthesis of *Monascus* pigments. The Mrpig P gene, which encodes an efflux transporter, is also observed in other filamentous fungi, such as *Aspergillus niger* (Zabala et al., 2012). This fact indicates that there is possibility of the efflux orange *Monascus* pigments into its extracellular broth during submerged culture. In fact, there is report that a hyper-pigment-producing mutant, derived from *Monascus* Kaoliang F-2 through a series of mutagenesis steps, produces pigments existing as lumps together with some viscous substances outside the cells (Lin and Lizuka, 1982). Recently, a collection of crystal *Monascus* pigments by further purification is reported



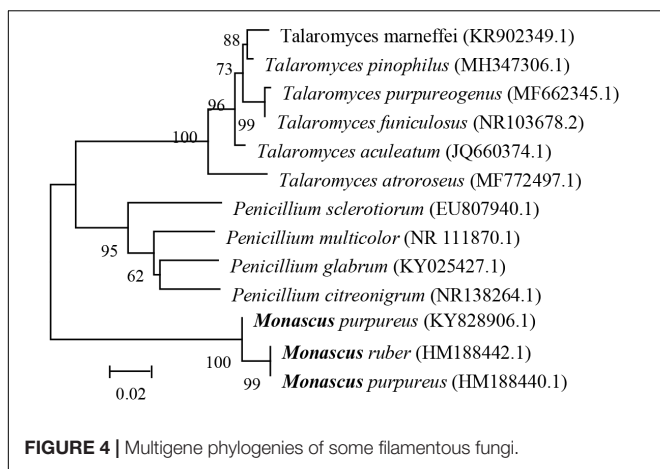
**FIGURE 3 |** Production of extracellular orange *Monascus* pigments. **(A)** Mycelia morphology of submerged culture (bar = 20  $\mu$ m); **(B)** needle-like crystals of orange *Monascus* pigments (bar = 50  $\mu$ m).

in literature (Vendruscolo et al., 2014) and production of extracellular *Monascus* pigments is also confirmed (Lu et al., 2018). The mycelia morphology of submerged culture is observed by optical microscope. There are many lumps of pigments sticking on the mycelia surfaces (**Figure 3A**). Those pigments are collected by membrane filtration, which is confirmed majorly as orange *Monascus* pigments (**1/2**) (**Figure 3B**) and a few of yellow *Monascus* pigments (**17/18**). This fact eliminates the long time mistaken concept that orange (yellow) *Monascus* pigments are predominantly cell-bound, including both intracellular and surface-bound pigments (Lin and Lizuka, 1982; Chen and Johns, 1994; Mapari et al., 2012). Some even manage to export the intracellular or cell-bound *Monascus* pigments during submerged culture by addition of non-ionic surfactant (Hu et al., 2012) or the antifungal agent fluconazole (Kolia et al., 2017). This mistake may be attributed to the confusion between water-insoluble *Monascus* pigments and intracellular ones.

## PRODUCTION OF *Monascus*-LIKE PIGMENTS

Filamentous fungi are large-scale producers of secondary metabolite with azaphilone structure. Orange *Monascus* pigments are a kind of secondary metabolites with azaphilone structure. Some *Talaromyces* species (Woo et al., 2014) or *Penicillium* species (Wei et al., 2017) also produce pigments with similar structure of *Monascus* pigments.

Internal transcribed spacer (ITS) sequence of *Penicillium* species, *Talaromyces* species, and *Monascus* species, were obtained from NCBI. Data set were aligned using MEGA5 software. The ITS sequences of thirteen strains were selected and aligned by ClustalW. Then, the unaligned parts at both ends were deleted. Finally, the aligned sequences were used for phylogenetic analysis. A phylogenetic tree with the ITS region of *P. sclerotiorum* FS50 was constructed by Neighbor-Joining method with 1000 bootstrap replication using MEGA5 software based on the distance matrix for all pairwise sequence combinations (**Figure 4**). *Talaromyces* species were highly homologous to *Penicillium* species. The homology between *Penicillium* species and *Monascus* species was more closed than that of *Talaromyces* species and *Monascus* species.

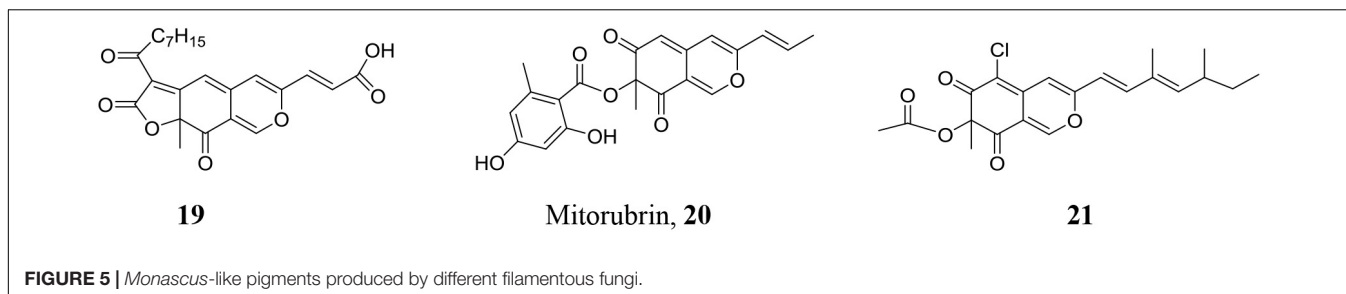


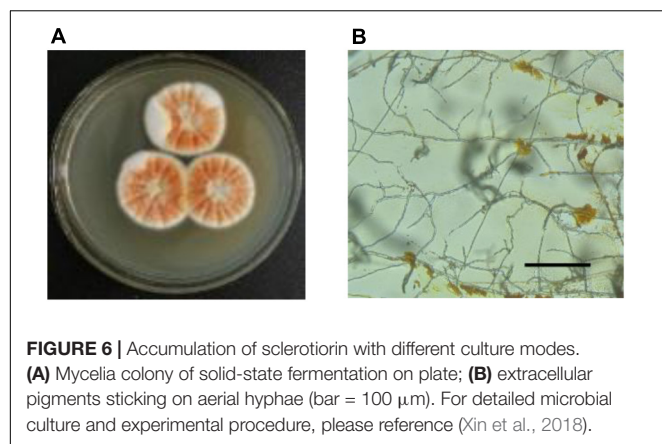
Just like secondary metabolites of *Monascus* species, *T. marneffei* (formerly known as *P. marneffei*) produces both *Monascus* pigments and citrinin (Woo et al., 2014). It indicates that orange *Monascus* pigments (1/2) can be produced by non-*Monascus* sp. However, *T. marneffei* is the most important thermal dimorphic fungus causing respiratory, skin and systemic mycosis (Woo et al., 2007), which is excluded from food microbiology for production of *Monascus* pigments. *P. purpureogenum* (formerly known as *P. purpureogenum*) produces *Monascus* pigment homolog (19 of Figure 5) besides the conventional *Monascus* pigments (Arai et al., 2015). *T. purpureogenus* is recognized to be interesting industrially (Mapari et al., 2012), but mycotoxins, such as rubratoxin A and B and luteoskyrin, are also produced (Frisvad et al., 2013). *T. atrovirens* produces mitorubrin (20) along with orange *Monascus* pigments without being accompanied by mycotoxin synthesis (Frisvad et al., 2013). Mitorubrin is a azaphilone chemical, which has similar structure to orange *Monascus* pigments except lack of lactone ring structure. Then, *T. atrovirens* is regarded as potential strain for replacement of *Monascus* sp. to produce pigments (Mapari et al., 2009). Those results indicate that *Monascus* pigment homologs (19) or *Monascus*-like pigments (20), even *Monascus* pigments, can be produced by *Talaromyces* species.

In spite of the close relationship between *Penicillium* species and *Monascus* species, there is no report on production of

*Monascus* pigments by *Penicillium* species. However, *Penicillium* species can be utilized for production of *Monascus*-like azaphilones. In 1940, sclerotiorin (21) was isolated and identified from the fermentation broth of *P. sclerotiorum* (Curtin, 1940). This is a *Monascus*-like pigment with the replacement of C-6 hydrogen with chlorine. Production of sclerotiorin as well as its derivatives by other *Penicillium* species is also reported (Hemtasin et al., 2016). Those *Penicillium* species do not co-produce citrinin or any other known mycotoxins and non-pathogenic to humans, which are potential strains for production of food pigments (Mapari et al., 2008; Dufossé et al., 2014; Gomes and Takahashi, 2016).

Traditionally, *Monascus* pigments are produced by solid-state fermentation on rice. In 1975, submerged culture was introduced into the production of *Monascus* pigments (Yoshimura et al., 1975). During submerged culture, mycelia of *Monascus* sp. acts as planktonic cells and extracellular *Monascus* pigments are produced (Figure 3A). However, sclerotiorin is usually produced by solid-state fermentation (Lin et al., 2015; Wei et al., 2017) or submerged culture without stirring (mycelia of *Penicillium* sp. acting as mycelial mat on air-liquid surface) (Curtin, 1940; Gomes and Takahashi, 2016). There are few reports on production of sclerotiorin by submerged culture with stirring (Celestino et al., 2014). Marine-derived strain *P. sclerotiorum* FS-50 (GenBank accession number EU 807940) was isolated from the sediment of South China Sea and stored in Guangdong Microbial Culture Collection Center (GDMCC) (Lin et al., 2015). Microbial fermentation in Czapek medium (agar 15g, glucose 20 g, NaNO<sub>3</sub> 3 g, KH<sub>2</sub>PO<sub>4</sub> 1 g, KCl 0.5 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.05 g, per liter of tap water with natural pH) was carried out in different modes for 7 days. In solid-state plate culture, yellow color was observed on the mycelial surfaces (Figure 6A). Further microscopic observation indicated that the yellow pigment was attached on aerial hyphae (Figure 6B). The production of extracellular pigments is very similar to that of orange *Monascus* pigments (Figure 3B). The extracellular yellow pigment was identified as sclerotiorin. Very interestingly, submerged culture in the Czapek medium in the absence of agar with stirring and without stirring exhibited very different characters. Mycelial mat formed on the air-liquid surface during submerged culture without stirring and the mycelial mat exhibited yellowish due to containing sclerotiorin. On the contrary, mycelia exhibited as planktonic cells during submerged culture with stirring and there was nearly no sclerotiorin accumulation. The





**FIGURE 6 |** Accumulation of sclerotiorin with different culture modes. **(A)** Mycelia colony of solid-state fermentation on plate; **(B)** extracellular pigments sticking on aerial hyphae (bar = 100  $\mu\text{m}$ ). For detailed microbial culture and experimental procedure, please reference (Xin et al., 2018).

mechanism behind the phenomenon remains blurry (Xin et al., 2018).

## CHEMICAL MODIFICATION OF *Monascus* PIGMENTS

Orange *Monascus* pigments (1, 2) as well as *Monascus*-like pigments (19, 20, 21) are the secondary metabolites of microbial fermentation. The chemical diversity is limited by the screened microbial strains. Fortunately, this subclass of azaphilones can be further modified chemically.

### Replacement of Pyranyl Oxygen With Primary Amine

Orange *Monascus* pigments (Figure 1) as well as *Monascus*-like pigments are a subclass of azaphilones with the characteristic reaction, i.e., replacement of pyranyl oxygen with primary amine form red pigments (Figure 7A). This characteristic reaction is known as aminophilic reaction in the following text. Aminophilic reaction begins with Michael addition of a nucleophilic primary amine to the electrophilic C-1 carbon and results in the formation of carbinolamine (22), in which involves electric transfer in the conjugated double bond system (red arrows). Chemical 22 undergoes C-O bond cleavage to generate enamines (23, 24), which involves electric transfer in the other conjugated double bond system (green arrows). An intramolecular proton transfers from nitrogen to oxygen gives enamine (25) followed by 26. Nucleophilic attack on the C-2 carbonyl by the lone electron pair of enamines (27) results in the formation of 28, which undergoes dehydration to give the nitrogen-containing orange *Monascus* pigment derivatives (OMPDS) with various primary amine residues (29) (Wei and Yao, 2005). In this possible mechanism, azaphilone structure of C-1 hydrogen, two sets of conjugated double bond systems (green arrows and red arrows) are necessary for the aminophilic reaction.

The mechanism of aminophilic reaction is confirmed by the reactive activity of some azaphilones (Figure 7B). Similar to orange *Monascus* pigments, replacement of pyranyl oxygen in sclerotiorin (21) with primary amine occurs smoothly while

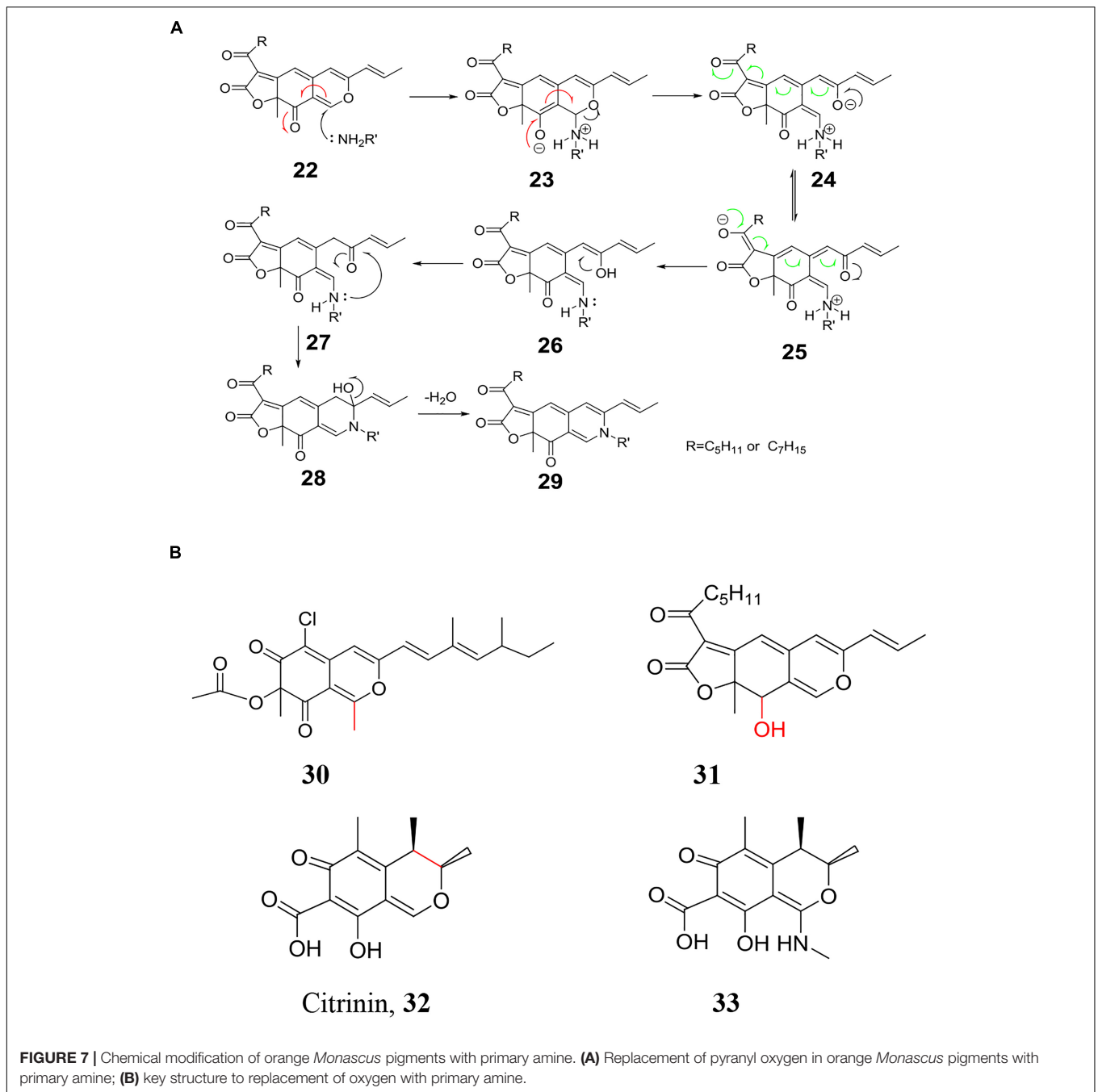
methylation of C-1 carbon in sclerotiorin (30) blocks this chemical reaction (Gomes and Takahashi, 2016). Hydroxylation of C-3 carbon (31) (Zheng et al., 2010) or cyclization of C-3 carbon to formation of *Monascus* metabolites with strong blue fluorescence (15, 16) (Huang et al., 2008) also prevents this chemical reaction to occur due to the lack of conjugated double bond system (red arrows in Figure 7A). With the same principle, replacement of pyranyl oxygen in yellow *Monascus* pigments (17, 18) with primary amine is also blocked due to the lack of conjugated double bond system (green arrows in Figure 7A). There is no chemical reaction between yellow *Monascus* pigments (17, 18) and primary amine, which has been applied for separation of yellow *Monascus* pigments from orange ones after microbial fermentation (Zhong et al., 2015). Similarly, primary amine also fails to replacement of oxygen in citrinin (32). However, it is also reported that methyl amine attacks the C-1 carbon to formation of new compound 33 (Natsume et al., 1988).

### Orange *Monascus* Pigment Derivates

Aminophilic reaction of orange *Monascus* pigments provides a chance to diversification of *Monascus* pigments due to the large number of primary amines. The well-known red *Monascus* pigments, rubropunctamine (34) and monascorubramine (35), are a pair of OMPDs with ammonia residue (Figure 8A). The primary amines of OMPDs (29) may be natural or non-natural primary amines. Many OMPDs with various natural primary amines, such as glucosamine (36, 37) (Hajaj et al., 1997), *L*-amino acids, such as *L*-tryptophan (38, 39) (Kim et al., 2007a), *L*-threonine (40, 41) (Jeun et al., 2008), and *L*-leucine (42, 43) (Sun et al., 2012), ethanol amine (44, 45) (Frisvad et al., 2013), even non-natural *D*-amino acid (46, 47) (Kim et al., 2007b), are isolated from fermentation broth. At the same time, OMPDs with various non-natural primary amines, such as 4-phenylbutylamine (48, 49) (Choe et al., 2012), are also achieved by direct chemical modification of orange *Monascus* pigments.

Compared to enlarging the chemical library by various primary amines (Figure 8A), replacement of orange *Monascus* pigments with *Monascus* pigment homologs or *Monascus*-like pigments may be a more efficient strategy (Figure 8B). Corresponding to *Monascus* pigment homolog (19), derivate with ammonia residue (50) is also isolated (Ogihara et al., 2000). A series of sclerotiorin derivatives with different non-natural primary amines are also prepared by chemical reaction between sclerotiorin and primary amines (Wei et al., 2017). Especially, a sclerotiorin derivate with phenylethylamine residue (51) had been produced by sequential fungal fermentation-biotransformation process (Gomes and Takahashi, 2016).

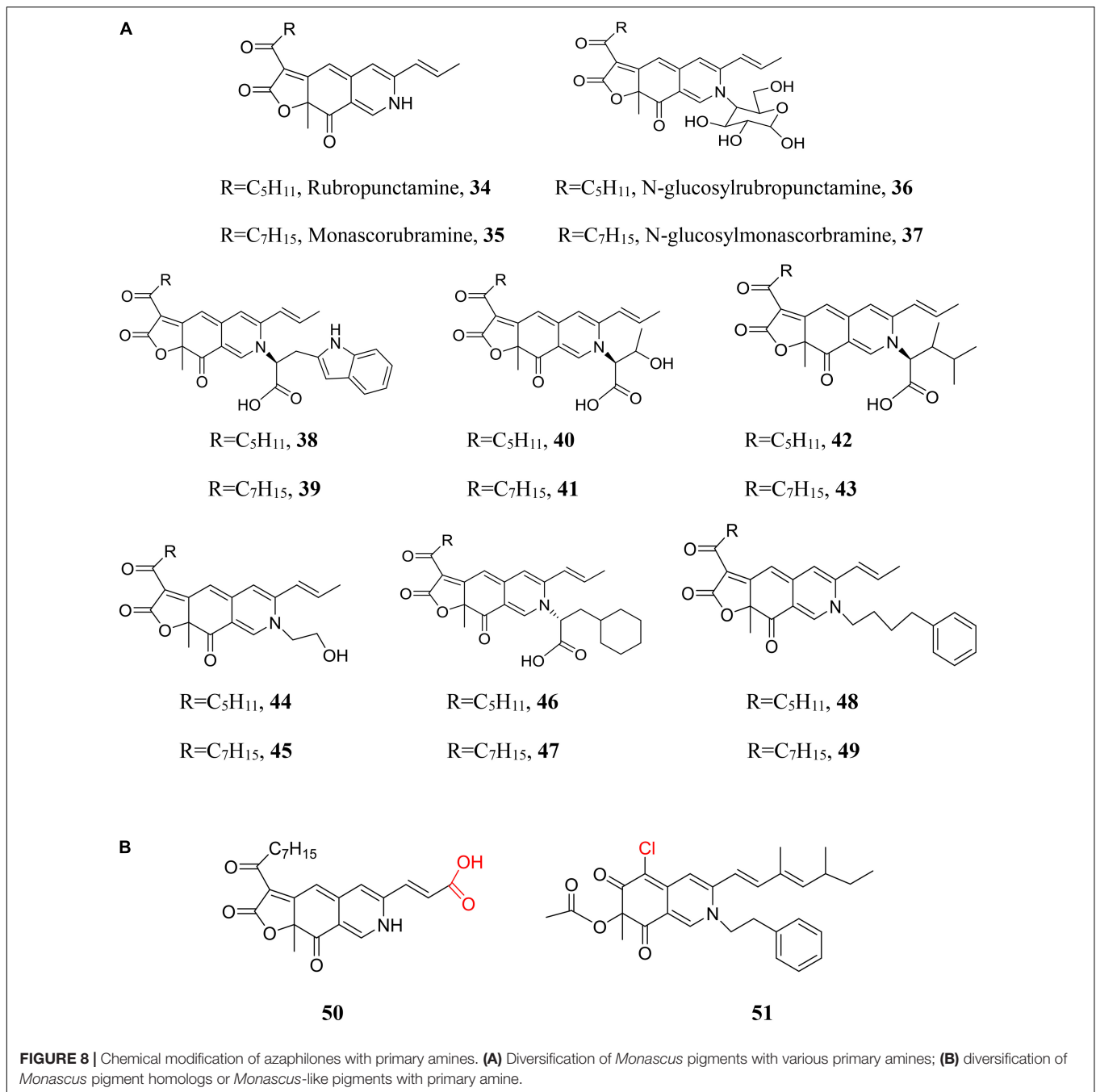
Preparation of OMPDs (29) involves biosynthesis of orange *Monascus* pigments and chemical modification of orange *Monascus* pigments with primary amine. One way is separation of orange *Monascus* pigments after *Monascus* fermentation and then carrying out chemical reaction between orange *Monascus* pigments with natural or non-natural primary amine. Method for solvent extraction of orange *Monascus* pigments from fermentation broth as well as the purification of orange *Monascus* pigments from the extractant by silica gel adsorption is suggested



(Choe et al., 2012). The chemical reaction involves the water-insoluble orange *Monascus* pigments and water-soluble primary amines. An ethanol aqueous solution (Lin et al., 1992), the organic solvent triethylamine (Wei et al., 2017), a non-ionic surfactant micelle aqueous solution (Xiong et al., 2015), even an aqueous solution with adsorbent (Achard et al., 2012) are utilized as reaction medium to enhance the heterogeneous reaction rate. Alternatively, OMPDs can also be produced directly by microbial fermentation. Red Yeast Rice is a traditional Chinese food colorants produced directly by solid-state fermentation of *Monascus* species on rice, in which red pigments (i.e., a

mixture with many kind of OMPDs) are produced due to the presence of a large number of amino acids or peptides in rice. However, whether involving enzymatic catalysis in the heterogeneous chemical reaction during the fermentation process remains unclear. Based on this fact, addition of a relatively excess of natural primary amines, such as amino acids, into the fermentation medium is applied for direct production of OMPDs (Blanc et al., 1994; Jung et al., 2003; Woo et al., 2014) and the OMPDs are further separated and purified by silica column chromatography (Jung et al., 2003). The chemical constituents of OMPDs are complicated due to the microbial



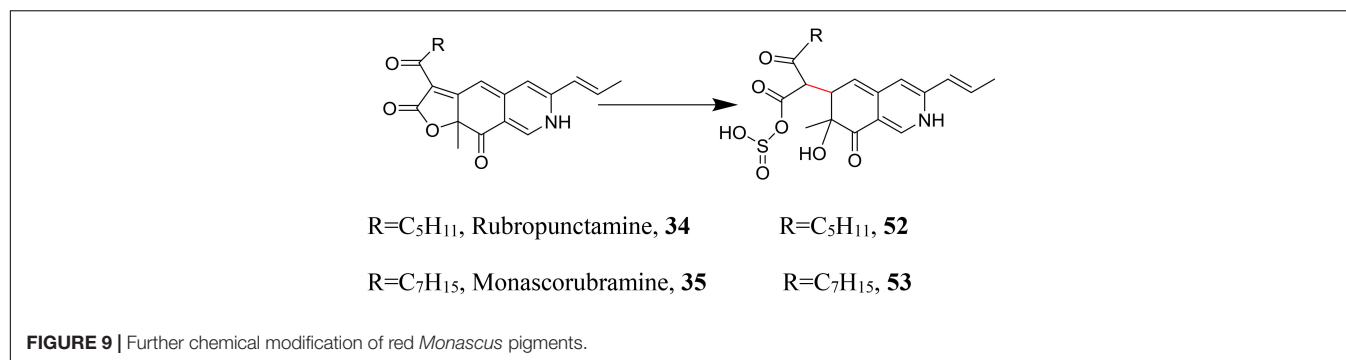


metabolism involving complex primary amines, which makes silica gel column chromatography only suitable for preparation of a small amount of sample.

## Characters of *Monascus* Pigment Derivates

Orange *Monascus* pigments are water-insoluble pigments with characteristic absorbance wavelength at approximately 470 nm in an ethanol aqueous solution. The low water-solubility limits the application of orange *Monascus* pigments as food colorant.

Even red *Monascus* pigments (**34**, **35**) remains a limited solubility in water and only solid state of Red Yeast Rice is utilized traditionally as food colorant. It is reported that replacement of ammonia with various amino acid residues strongly alters the hydrophobicity of OMPDs. At the same time, the characteristic absorbance wavelength also transfers from red *Monascus* pigments (**34**, **35**) at approximately 508 nm to varying from 498 to 525 nm depending on a special amino acid (Jung et al., 2003). These characters are further confirmed by examination of the solubility of OMPDs with water-soluble amino acids (Wong and Koehler, 1983). Furthermore, the



stability of OMPDs is also influenced by the primary amine structure. It is well known that photo-instability is the drawback of *Monascus* pigments (Sweeny et al., 1981). The photo-stability of OMPDs with various amino acid residues is enhanced markedly under sunlight irradiation condition compared to the red *Monascus* pigments (**34**, **35**). More interestingly, OMPDs with amino acid residues exhibit stable at nearly neutral pH while red *Monascus* pigments (**34**, **35**) are relatively stable under acidic condition (Jung et al., 2005, 2011). At the same time, thermal stability of OMPDs is enhanced compared to orange *Monascus* pigments themselves (Vendruscolo et al., 2013). Red pigments produced by *P. purpurogenum* GH2 also shows a relatively higher thermal stability (Morales-Oyervides et al., 2015). The characters of red color, water-soluble, and high stability at neutral pH makes OMPDs with amino acid residues potential of food colorant. The food additives standard of a red colorant has already been issued and updated several times by government of the People Republic of China (Red *Monascus* Pigments<sup>®</sup>, GB1886.181-2016). The major constituents of Red *Monascus* Pigments<sup>®</sup> are OMPDs with various amino acid/peptide residues.

Native yellow *Monascus* pigments (**17**, **18**) are hydrophobic/water-insoluble with its characteristic absorbance wavelength approximately 400 nm. Very luckily, Red *Monascus* pigments<sup>®</sup> can be further chemically modified with sodium hydrosulfite to produce water-soluble yellow pigments. Novel compounds (**52**, **53**) are isolated and identified from the water-soluble yellow *Monascus* pigments, which exhibits characteristic absorbance wavelength at 468 nm. Chemical transformation of red *Monascus* pigments (**34**, **35**) into compounds (**52**, **53**) by reduction and sulfonation reaction (**Figure 9**) may be involved in the chemical modification of Red *Monascus* Pigments<sup>®</sup> with sodium hydrosulfite (Yang et al., 2018). The safety of this water-soluble yellow *Monascus* pigments is also evaluated and the corresponding food additive standard has been issued recently by the Chinese government (Yellow *Monascus* Pigments<sup>®</sup>, GB 1886-66-2015).

## **Monascus** PIGMENTS ACTING AS ENZYME INHIBITOR

Red Yeast Rice as a traditional Chinese medicine involves many bioactive constituents (Ma et al., 2000). At the same time,

*Monascus* pigments are series of azaphilones. Azaphilones are a class of fungal metabolites with diverse bioactivities (Osmanova et al., 2010). There are many reports about the bioactivity of *Monascus* pigments, such as the antimicrobial activities of OMPDs with amino acid residues (Kim et al., 2006) and the anti-cancer activity of *Monascus* pigments (Zheng et al., 2010; Yang et al., 2014), as well as *Monascus*-like pigments, such as the antifouling activity of the sclerotiorin derivatives (Wei et al., 2017). We restricted the scope within pigment bioactivity acting as enzyme inhibitors in the present work.

## **Pancreatic Lipase Inhibitor and Obesity**

Physical inactivity and overeating are the main causes of obesity. Hence, controlling the digestion of dietary lipids is a promising approach to treat obesity. Lipstatin is a  $\beta$ -lactone molecule which controls the digestive activity of pancreatic lipases and thus controls the fat absorption in the small intestine, which is utilized as anti-obesity medicine (Kumar and Dubey, 2015). On the other hand, differentiation and hypertrophy of adipocytes are fundamental processes of obesity. The differentiation of pre-adipocytes into adipocytes involves exposure of a confluent, quiescent population of cells to a variety of effectors that activate a cascade of transcription factors. This cascade begins with the CCAAT/enhancer-binding protein (C/EBP)  $\beta$  and C/EBP  $\delta$ , which finally induce the expression of C/EBP  $\alpha$  and peroxisome proliferator activated receptor (PPAR)  $\gamma$ . These transcription factors coordinate the expression of genes involved in creating and maintaining the adipocyte phenotype (Rosen et al., 2000).

Orange *Monascus* pigment derivatives with different amino acid residues, such as unnatural amino acids (Kim et al., 2007b), L/D-amino acids (Kim et al., 2007a), are screened for pancreatic lipase inhibitor. OMPD with *L*-tryptophan residue (**38**, **39**) exhibits the highest inhibitory activity with  $\text{IC}_{50}$  value 61.2 mM (Kim et al., 2007a). Administration of the jelly food with OMPDs (**38**, **39**) also confirms that the total cholesterol, LDL (low-density lipoprotein) cholesterol and triacylglycerol levels in the mouse serum are lowered. Significant reduction of subcutaneous fat and visceral fat amounts in mice are also observed using micro CT images of mice tissues (Nam et al., 2014). In addition, experiments also found that OMPDs with 4-phenylbutylamine (PBA, **48/49**) or 2-(*p*-toyl) ethylamine (TEA) residue also shows an inhibitory activity against adipogenic differentiation in 3T3-L1

cells. The transcription factors PPAR  $\gamma$  and C/EBP  $\alpha$  are down-regulated by the PBA derivative and the TEA derivative at 10  $\mu$ M. Both the number and droplet size of fatty cells are reduced by treatment with the inhibitory derivatives (Choe et al., 2012).

Besides OMPDs exhibiting as anti-obesity agents, yellow *Monascus* pigments, ankaflavin (17) and monascin (18), exhibit anti-obesity effect via the suppression of differentiation and lipogenesis (Lee et al., 2013). Red Yeast Rice with high content of yellow *Monascus* pigments, market known as Ankascin<sup>®</sup>, has already been commercialized and approved by the US FDA as a new dietary ingredient (NDI). The anti-obesity effect of yellow *Monascus* pigments is related to down-regulate the transcription factors C/EBP  $\beta$ /PPAR  $\gamma$  expression, inhibit lipogenesis by increasing lipase activity, and suppress Niemann-Pick C1 Like 1 (NPC1L1) protein expression associated with small intestine tissue lipid absorption (Jou et al., 2010; Lee et al., 2013). The effect of yellow *Monascus* pigments on obesity-related-diseases is also investigated, such as hyperlipidemia, i.e., down-regulation of total cholesterol, triglyceride, LDC cholesterol level in the serum, and up-regulation of low-density lipoprotein (HDL) cholesterol level in serum (Lee et al., 2010), steatohepatitis (Hsu et al., 2014), and hyperglycemia (Hsu et al., 2013).

## HMG-CoA Reductase Inhibitor and Hyperlipidemia

Anabolism of cholesterol occurs by transfer of acetyl-CoA from the mitochondrion to the cytosol. Acetyl-CoA can be sequentially converted to hydroxy-methyl-glutaryl coenzyme (HMG-CoA), mevalonate, squalene, lanosterol, and cholesterol. The conversion reaction of HMG-CoA to mevalonate, which is catalyzed by HMG-CoA reductase, is known to be a key rate-limiting step in cholesterol biosynthesis. Activity regulation of HMG-CoA reductase can control the cholesterol content in the body and then hypercholesterolemia. Lovastatin, produced by fermentation of *Aspergillus terreus* (Boruta and Bizukojc, 2017) as well as *Monascus* sp. (Lu et al., 2013), is a well-known inhibitor of HMG-CoA reductase and widely used as a hypercholesterolemia drug for reduction of plasma cholesterol levels in humans.

Orange *Monascus* pigment derivatives with different amino acid residues are further screened for HMG-CoA reductase inhibitor (Jeun et al., 2008). Using orange *Monascus* pigments (1, 2) as control (exhibiting high inhibitor activity to HMG-CoA reductase), experimental result indicates that OMPDs with threonine residue (40, 41) have good exhibitory activity (38%) while derivatives with other amino acid residues shows low activity compared to orange *Monascus* pigments. *In vivo* tests using female C57BL/6 mice further confirms the total cholesterol level of mouse serum is reduced by 8–9% with OMPDs (40, 41) and by 16% with orange *Monascus* pigments. Supplementation with OMPDs (40, 41) and orange *Monascus* pigment decreases the LDL cholesterol level by 18–26% and increases the HDL cholesterol level by 1–9%.

Cellular lipid and cholesterol metabolism play either a direct or indirect role in membrane integrity. In particular, cholesterol

is proposed as an integral part of lipid raft structure (Garcia-Fernandez et al., 2017). The replication of hepatitis C virus (HCV) depends on the host cells to provide cholesterol as raw materials. A block of mevalonate biosynthesis pathway in host cells should inhibit HCV replication. Orange *Monascus* pigments as well as their amino acid derivatives are further screened for HCV antiviral agent. A group of OMPDs with various amino acid residues, such as leucine (42, 43), significantly inhibit HCV replication (Sun et al., 2012). Similarly, lipid rafts in cell membrane of pathogen methicillin resistant *Staphylococcus aureus* (MRSA) is also related to its multidrug-resistant. Interfering with cholesterol biosynthesis pathway is also potential for dealing with multidrug-resistant of MRSA (Garcia-Fernandez et al., 2017).

## PTP1B Inhibitor and Hyperglycemia

Anti-diabetic thiazolidinedione (TZD) drugs, such as rosiglitazone and pioglitazone, are PPAR- $\gamma$  agonists and used to manage obesity-related insulin resistance and type 2 diabetes (hypoglycemic agent). Alternatively, protein-tyrosine phosphatases (PTP) have an important role in the regulation of insulin signal transduction. PTP1B, protein tyrosine phosphatase 1B, is a prototype non-receptor cytoplasmic PTP enzyme that negatively regulates insulin and leptin signaling pathways (Ahmad et al., 1997). Thus, PTP1B inhibitor is a potential agent for the treatment of diabetes.

An interesting paper, using a new method of ultrafiltration-based LC-MS to directly screening of PTP1B inhibitor from the traditional Red Yeast Rice, has been published recently. The experimental result shows that monascorubramine (35) possesses inhibitory activity toward PTP1B and the anti-diabetic effect of Chinese Red Yeast Rice is partially attributed to potential PTP1B inhibitory activity of monascorubramine (Jin et al., 2016). Monascorubramine acting as PTP1B inhibitor is based on the chemical library of native Red Yeast Rice. It reasonably deduces that there is more efficient PTP1B inhibitor in the library of bio-and chemo-diversification of native *Monascus* pigments as well as *Monascus*-like pigments (Figure 8).

## SUMMARY

Traditionally, *Monascus* pigments are produced natively by solid-state microbial fermentation on rice. The Red Yeast Rice has been utilized as food colorant and traditional Chinese medicine for more than 1000 years (Ma et al., 2000; Chen et al., 2015). The safety of Red Yeast Rice has been confirmed by Chinese as well as other East Asia people. Due to the complex secondary metabolites, such as citrinin (32), Red Yeast Rice is still be excluded in the list of food additives by the European Food Safety Authority (EFSA) (Kallscheuer, 2018).

With the progress on study of the biosynthetic pathway of *Monascus* pigments, it is recognized that red pigments of *Monascus* fermentation result from the chemical modification of orange *Monascus* pigments with different primary amines. By application of submerged culture other than traditional solid-state fermentation technique, Red *Monascus* Pigments<sup>®</sup>, the

major constituents are OMPDs with various amino acid/peptide residues, can be produced commercially. In the updated food additive standard of Chinese government (GB1886.181-2016), the content of citrinin is also strictly restrained. The utilization of red colorant from filamentous fungi in food industry is the reality (Dufosse, 2018). With the further progress on chemical modification of orange *Monascus* pigments, production of OMPDs (29) with a single primary amine residue becomes possible. The red colorants from filamentous fungi acting as food additives will be finally approved by more and more countries.

Many bioactive components have been isolated and identified from the Chinese traditional medicine Red Yeast Rice. Yellow *Monascus* pigments exhibit anti-obesity activity as well as obesity-related-diseases, such as hyperlipidemia, steatohepatitis, and hyperglycemia (Hsu and Pan, 2014). Red Yeast Rice with high content of yellow *Monascus* pigments, market known as Ankascin®, has been approved by the US FDA as a

new dietary ingredient (NDI). With the diversification of the chemical structures of orange *Monascus* pigments/*Monascus*-like pigments (Figure 8), a very large chemical library should provide more chances for screening bioactive compounds. Besides yellow *Monascus* pigments, it is foreseeable that more and more OMPDs (29) will be added into the list of functional food.

## AUTHOR CONTRIBUTIONS

LL wrote the section of “Introduction”, “Biosynthesis of *Monascus* Pigments”, and “*Monascus* Pigments Acting as Enzyme Inhibitor”. YH wrote the section of “Chemical Modification of *Monascus* Pigments”. QX wrote the section of “Production of *Monascus*-Like Pigments”. JZ and ZW read the whole manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Production of an Enzymatic Extract From *Aspergillus oryzae* DIA-MF to Improve the Fructooligosaccharides Profile of Aguamiel

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Aguamiel is a natural sap produced by some species of agave plants, such as *Agave salmiana*, *A. atrovirens*, or *A. angustifolia*. It is a product with a high concentration of fructose, glucose or sucrose, although its composition may vary depending on the season in which it is produced, and may also contain agave fructans (or agavins) or fructooligosaccharides (FOS). It has been reported that FOS can be produced by enzymes that act on sucrose or inulin, transfructosylating or hydrolyzing these materials, respectively. Due to the sugar content in aguamiel, the application of an enzymatic complex produced by *Aspergillus oryzae* DIA MF was carried out. This complex was characterized by 1-D electrophoresis SDS-PAGE, and its transfructosylation and hydrolysis activities were determined by HPLC. In order to determine the conditions at which the concentration of FOS in this beverage increased, kinetics were carried out at different temperatures (30, 50, and 70°C) and times (0, 1, 2, 3, 4, 5, 10, and 15 h). Finally, the antioxidant and prebiotic activities were evaluated. FOS concentration in aguamiel was increased from  $1.61 \pm 0.08$  to  $31.01 \pm 3.42$  g/L after 10 h reaction at 30°C applying 10% enzymatic fraction-substrate (v/v). Antioxidant activity was highly increased (34.81–116.46 mg/eq Trolox in DPPH assay and 42.65 to 298.86 mg/eq Trolox in FRAP assay) and growth of probiotic bacteria was higher in aguamiel after the enzymatic treatment. In conclusion, after the application of the enzymatic treatment, aguamiel was enriched with FOS which improved antioxidant and prebiotic properties, so it can be used as a functional food.

**Keywords:** agavins, antioxidant, fructooligosaccharides, fructosyltransferase, inulinase, prebiotic

## INTRODUCTION

Fructooligosaccharides (FOS) are oligosaccharides composed of fructose monomers linked to a glucose with a polymerization degree (DP) from three to ten; these oligosaccharides have  $\beta$ -2,1 or  $\beta$ -2,6 linkages in its structure. If the DP goes  $> 10$  these polysaccharides belong to the fructans group, and depending on its structure, they are named differently: inulin if the fructan structure has  $\beta$ -2,1 linkages, levan if it has  $\beta$ -2,6 linkages and, a fructan with both linkages in its structure is known as agavin (1).

FOS can provide different effects on human health. Due to the linkages they have, FOS can be eaten and reach the intestine intact because the digestive system cannot metabolize them; once they reach the intestine, the microbiota uses them as a substrate, classifying them as prebiotics (2). After these compounds are used by the microbiota as a substrate, bacteria like *Lactobacillus* or *Bifidobacterium* produce compounds to regulate cholesterol and triglycerides, increasing the mineral absorption in the intestine, and to regulate the production of diverse cytokines and immunoglobulin A, reducing the risk of developing colon cancer. It has also been reported that some FOS from the grain of *Coix lachryma-jobi* Linn can bring antioxidant activity 0.97-fold higher than of vitamin C (2–6).

FOS can be found in natural materials (e.g., onions, bananas, chicory), but its concentration is very low and by different extraction methods, the yield can be 0.009 g of FOS/ g of raw material (7). An alternative in FOS production is the employment of biotechnological processes. Some fungi and bacteria are able to produce enzymes with FOS production capacity, but fungi are mainly used due to their capacity to produce extracellular enzymes, making the production in culture media easier. Fungi can produce FOS by different enzymatic mechanisms: fructosyltransferases, which form FOS from sucrose by transfructosylation (8);  $\beta$ -fructofuranosidases with hydrolytic and fructosyltransferase activity, being the hydrolytic activity preponderant during transfructosylating (9); and inulinases, enzymes that hydrolyze inulin, producing fructose monomers (exoinulinase) or/and producing shorter inulin chains or FOS (endoinulinase) (10).

Infant formulas, pastry, confectionary, beverages, other food products and food supplements have been enriched with FOS. Nevertheless, the suggested minimum daily effective intake required to produce a beneficial health effect is 3–8 g FOS/portion of product (11). Agave plants can produce an exudate denominated aguamiel. Aguamiel is a liquid product rich in sugars, which are mainly composed of fructose, sucrose, FOS, and agavins; but, it also contains glucose and some maltooligosaccharides (12, 13). The concentration of FOS in aguamiel is low, but it's a material with great conditions before use as a product enriched with FOS, it has high concentrations of sucrose and agavins used to be hydrolyzed to produce more FOS. Also, aguamiel is a cheap product with a price lower than \$1 dollar and in its composition is made up of saponins, minerals and essential amino acids, along with the sugars it contains (14–16). The aim of this work was to increase the FOS concentration in aguamiel, by using an enzymatic complex produced by *Aspergillus oryzae* DIA-MF, to improve its antioxidant and prebiotic properties.

## METHODOLOGY

### Reagent and Standards

FOS standards 1-kestose (GF2), 1-nystose (GF3), and 1F-fructofuranosyl-nystose (GF4) were purchased from Wako Pure Chemical Industries, Ltd. (Japan Company). Fructose (F), glucose (G), and sucrose (S) standards were obtained from

Sigma Aldrich (St. Louis, MO, USA). A Trolox standard was obtained from Sigma Aldrich (St. Louis, MO, USA). Aguamiel was obtained from “Ejido Las Mangas” (long.  $-101.102500$ , Lat.  $24.906111$ ), Saltillo, Coahuila, Mexico from *Agave salmiana* in December 2017. It was sterilized by membrane filtration ( $0.45 \mu\text{m}$ ) before its use. Orafti HSI<sup>®</sup> was obtained from Beneo Company (Belgium). Agavins were obtained from Megafarma<sup>®</sup> (Durango, Mexico). Protein marker Prestained SDS-PAGE standard #1610318 was obtained from Bio-Rad (Hercules, CA, USA). Silver BULLit<sup>™</sup> silver stain kit was obtained from AMRESCO (Radnor, PA, USA).

### Evaluation and Selection of the Crude Enzymatic Extract

Three different carbon sources (Orafti HSI<sup>®</sup> and Agavins at 60 and 120 g/L, and aguamiel) were used to produce the enzymatic extracts which were applied to aguamiel to evaluate the increase in FOS concentration. These enzymatic extracts were produced by *A. oryzae* DIA-MF at 0, 6, 12, 18, 24, 30, 36, and 48 h of fermentation, giving a total of 40 crude enzymatic extracts. For each extract the enzymatic activity was evaluated over aguamiel as follows: in 2 mL Eppendorf tubes 900  $\mu\text{L}$  of aguamiel were deposited, 100  $\mu\text{L}$  of extract were added to aguamiel and incubated at  $30^\circ\text{C}$  for 20 min. The reaction was stopped at  $100^\circ\text{C}$  for 3 min. All samples were filtered through  $0.45 \mu\text{m}$  nylon membranes. Samples were analyzed by high-performance liquid chromatography (HPLC, Perkin Elmer Series 200) using a Prevail Carbohydrate ES Column ( $5 \mu\text{m}$ ,  $250 \times 4.6 \text{ mm}$ , Grace) at  $30^\circ\text{C}$ . A mixture of acetonitrile/water 70:30 (v/v) and  $\text{NH}_4\text{OH}$  0.04% was used as a mobile phase at a flow rate of 1 mL/min with a pressure of 1,700 psi. A refractive index detector (RID) was operated at  $35^\circ\text{C}$ . The response of the RID was recorded and integrated using the TOTALCHROM WS V6.3 software. The quantification of FOS in samples was determined by using standards curves made with different known concentrations (17). The extract that showed the greatest increase in FOS concentration in aguamiel was selected for further steps.

### Fractioning of the Selected Enzymatic Extract

The selected extract was fractioned into four. The fractioning was made by a successive filtration with different membranes. The first membrane used to microfilter was a 450 kDa cellulose membrane in a Millipore vacuum glass system. After microfiltration, the extract was ultrafiltered in an Amicon Stirred Cell<sup>®</sup> (Millipore) in a nitrogen atmosphere, through 300, 100, and 30 kDa nylon membranes (Millipore) consecutively.

### Transfructosylase, Hydrolase, and Inulinase Activity of the Fractions

Hydrolytic and transfructosylating activity evaluated in the four fractions was performed in a 2 mL Eppendorf tube, 900  $\mu\text{L}$  of sucrose solution (4% in acetate buffer, pH 4.5, 50 mM) were deposited, and 100  $\mu\text{L}$  of the enzymatic extract fraction was added. For inulinase activity, in a 2 mL Eppendorf tube 900  $\mu\text{L}$  of inulin solution (1% in acetate buffer, pH 4.5, 50 mM)



were deposited and 100  $\mu$ L of the enzymatic extract fraction was added. The reactions were performed for 20 min at 30°C. The reaction was stopped at 100°C for 3 min. The three enzymatic activities of each fraction were analyzed in HPLC following the methodology described above (section Evaluation and Selection of the Crude Enzymatic Extract). One enzymatic unit of hydrolase (Uh) was determined as the enzyme necessary to liberate 1  $\mu$ mol of glucose per minute; one enzymatic unit of transfructosylase (Ut) was determined as the enzyme necessary to produce 1  $\mu$ mol of kestose per minute; and one enzymatic unit of inulinase (Ui) was determined as the enzyme necessary to liberate 1  $\mu$ mol of kestose per minute, all under certain conditions (10, 17). Protein of every fraction was measured following Bradford's method (18). The selection of the fraction was determined by the highest activity in FOS production.

### Electrophoresis of the Fractions

The selected fraction was analyzed by electrophoresis. It was performed in a miniProtean<sup>®</sup> Tetra-cell chamber. Twenty microliters of sample were added to the wells and 8  $\mu$ L of the pre-stained marker #1610318. The running buffer was added and the electrophoresis was performed at 80 V for 30 min and later at 120 V for 1 h. The gels were stained with silver staining technique with the Silver BULLit<sup>™</sup> kit, following the fabricant instructions.

### Enzymatic Kinetic in Aguamiel

The enzymatic kinetic of the selected fraction was performed in 2 mL Eppendorf tubes with 900  $\mu$ L of aguamiel, and 100  $\mu$ L of the selected fraction were added to every tube. The kinetics were performed at 30, 50, and 70°C, and samples were withdrawn at 1, 2, 3, 4, 5, 10, and 15 h of reaction. The reactions were stopped at 100°C for 3 min. The FOS analysis of the samples was performed by HPLC following the methodology described above (section Evaluation and Selection of the Crude Enzymatic Extract) (17).

### Antioxidant Activity

The antioxidant activity of aguamiel before and after enzymatic treatment was performed by three methods: DPPH and FRAP assays. Antioxidant capacity was determined by a standard curve prepared with a Trolox standard.

### DPPH Radical Scavenging Activity

The DPPH<sup>·</sup> assay was carried out according to the methodology reported by Molyneux (19). Seven microliters of each sample were placed in a microplate, and 193  $\mu$ L of 60  $\mu$ M DPPH radical solution were added. The microplate was placed in the dark for 30 min and the absorbance was measured at 517 nm.

### FRAP Assay

Ferric ion reducing antioxidant power assay was carried out according to the methodology reported by Benzie and Strain (20). A 10 mM TPTZ in 40 mM HCl solution was mixed with a 20 mM FeCl<sub>3</sub> solution and 0.3 M sodium acetate buffer in 1:1:10 (v/v/v) proportions. Ten microliters of each sample were placed in a microplate and 290  $\mu$ L of the mixture solution were added to each sample. The reaction was placed in the dark for 15 min and the absorbance was measured at 593 nm.

**TABLE 1** | Evaluation of enzymatic extracts for FOS production in aguamiel.

Carbon source	Crude extract used for FOS production (g/L)			
	24 h	30 h	36 h	48 h
Agavins (60 g/L)	NP	NP	0.14 $\pm$ 0.02	0.11 $\pm$ 0.02
Agavins (120 g/L)	NP	NP	1.99 $\pm$ 0.11	2.26 $\pm$ 0.07
Aguamiel	0.13 $\pm$ 0.02	1.04 $\pm$ 0.12	NP	NP

*Orafti HSI<sup>®</sup> results are not included because these extracts did not show FOS production at any of evaluated times.*

### Prebiotic Activity of Aguamiel Before and After Enzymatic Treatment

Prebiotic activity was performed over *Lactobacillus plantarum* 14917, *Lactobacillus paracasei* 25302, *Bifidobacterium lactis*, and *Bifidobacterium bifidum* 450B, all strains obtained from the culture collection of the Food Research Department of Autonomous University of Coahuila (Mexico). All the strains were previously activated in MRS media culture at 37°C for 24 h. The kinetic growth of the strains was performed in a sterile 96 wells microplate. Aguamiel treated with the enzymatic fraction extract, crude aguamiel, and MRS media culture as a control were added to the microplate. Each strain was added to the three-different media to a final volume of 200  $\mu$ L per well with a concentration of  $1.5 \times 10^8$  colony forming units of bacteria. All kinetics were monitored each hour for 24 h spectrophotometrically by recording the OD<sub>600</sub> variations at 37°C (21). All kinetics were performed by triplicate.

### Statistical Analysis

All experiments were carried out by triplicate. All data were analyzed by a comparison of means. The variance analysis was performed in the Statistica 7 software using the Tukey's range procedure.

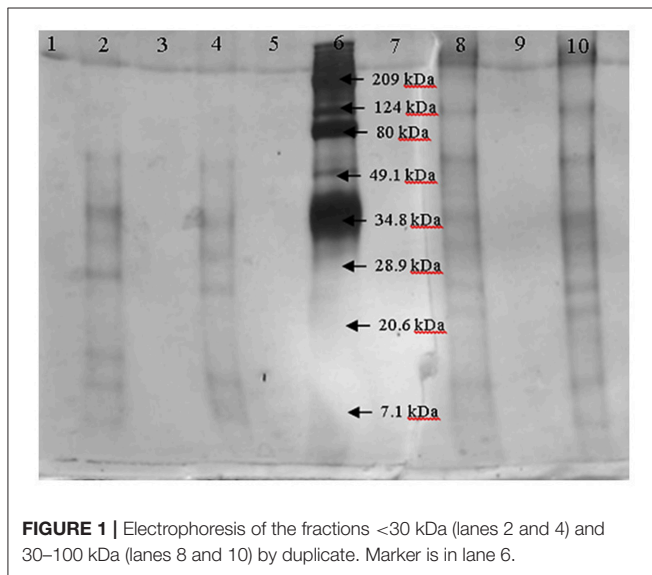
## RESULTS

### Crude Enzymatic Extracts Evaluation

The application of the 40 crude extracts showed that only six of them produced FOS using aguamiel as a substrate. All extracts obtained from the fermentation with Orafti HSI<sup>®</sup> as carbon source at both concentrations (60 and 120 g/L) did not show FOS production, as well as the extracts obtained using aguamiel and agavins as a carbon source between 0 to 18 h of fermentation. In **Table 1**, the extracts that produced FOS using aguamiel as a substrate are shown. Four of these six extracts were obtained from fermentation with agavins and the other two from aguamiel; the production of FOS was higher with the extracts obtained with agavins at a high concentration (120 g/L) as a carbon source. Furthermore, according to results, the extract obtained with agavins at 120 g/L and 48 h of fermentation showed the highest FOS production (2.26  $\pm$  0.07 g/L). Thus, this extract was selected for further steps.

**TABLE 2** | Enzymatic and specific activity of the four different fractions.

Fraction	Enzymatic activity			Specific activity		
	Hydrolytic (Uh/L)	Transferase (Ut/L)	Inulinase (Ui/L)	Hydrolytic (Uh/mg)	Transferase (Ut/mg)	Inulinase (Ui/mg)
300–450 kDa	0	309.20	0	0	77.11	0
100–300 kDa	0	299.72	0	0	74.74	0
30–100 kDa	1799.79	1473.15	0	488.81	367.39	0
<30 kDa	699.45	220.07	443.64	367.36	54.88	110.63

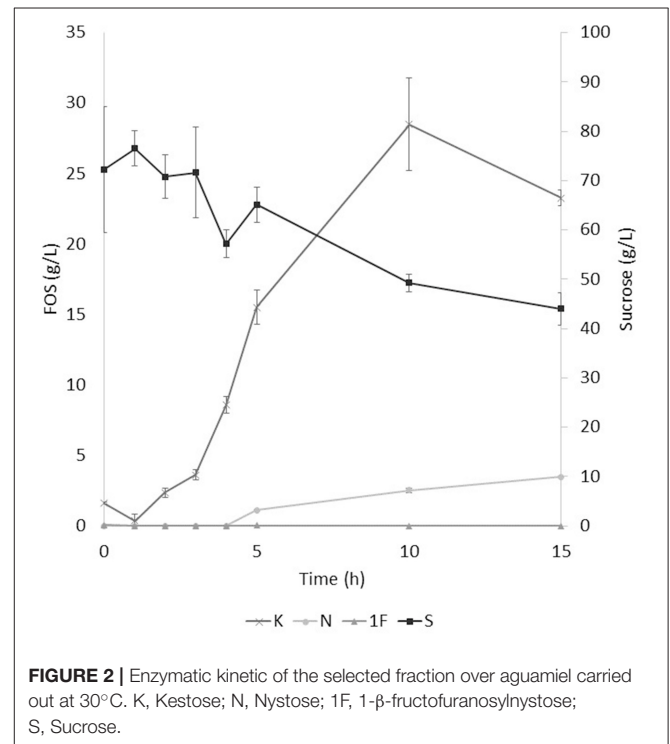


## Fractioning and Enzymatic Activities

After fractioning the selected crude extract, the three activities measured showed different performances. In **Table 2**, all the enzymatic activities of the four fractions are shown. Transferase activity was present in the four fractions, but the highest activity was shown in the 30–100 kDa fraction, with an activity almost five times greater than that of the other fractions. Hydrolytic activity was only present in the lower size fractions and the inulinase activity was only present in the <30 kDa fraction. Due to the enzymatic activities shown by the four fractions, the 30–100 kDa fraction was selected to be analyzed by electrophoresis and the <30 kDa fraction was analyzed to compare both fractions and analyze which protein could be responsible for inulinase activity (present only in <30 kDa fraction). In **Figure 1**, the electrophoretic profile of both fractions is shown; the fraction <30 kDa depicted four different proteins with molecular weights of ~7, ~11, ~29.5, and ~34 kDa. The 30–100 kDa fraction showed proteins with molecular weights of ~23, ~26.5, ~32, ~35, ~65, and ~124 kDa.

## FOS Production

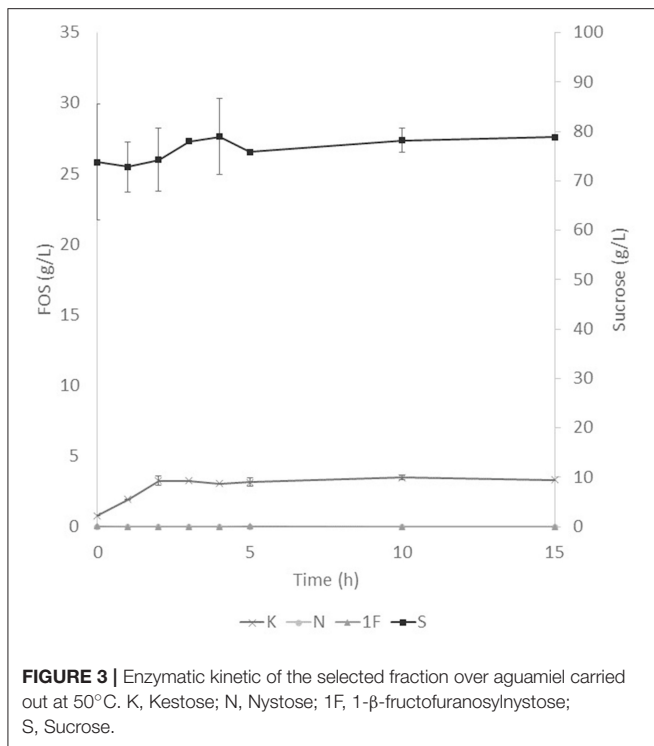
The application of the selected fraction over aguamiel showed better results in the 30°C kinetic (**Figure 2**). The 30°C kinetic showed the formation of FOS along the 15 h of the kinetic, but the highest concentration of FOS was achieved at 10 h with 31.01



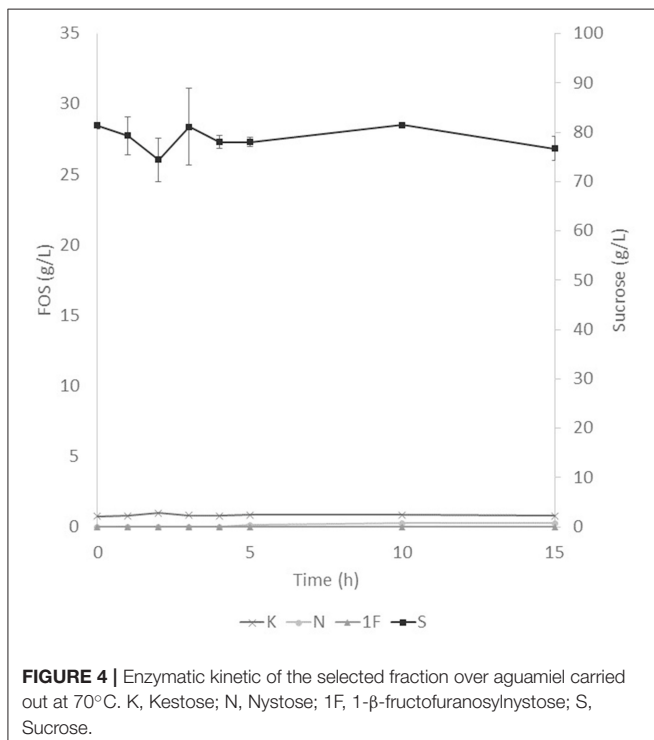
± 3.42 g/L total FOS concentration in aguamiel. In comparison with the initial concentration of 1.61 ± 0.08 g/L, it is almost 20-fold the initial concentration of FOS. Also, the kinetic depicted the standard FOS formation by transfructosylating activity, developing the formation of kestose and then the formation of nystose. The 50°C kinetic showed an increase in kestose concentration in the first 2 h (**Figure 3**), but after this time FOS concentration remained constant, without increasing any of the three FOS measured. Kinetic at 70°C did not show results or changes within the 15 h of the reaction (**Figure 4**).

## Antioxidant Activity

The antioxidant assay showed a difference between the aguamiel before and after the enzymatic treatment. DPPH and FRAP assays showed higher antioxidant activity on aguamiel after the enzymatic treatment; the difference was three times the antioxidant activity compared to the aguamiel without treatment in DPPH assay, and over five times in the FRAP assay, as shown in **Table 3**.



**FIGURE 3 |** Enzymatic kinetic of the selected fraction over aguamiel carried out at 50°C. K, Kestose; N, Nystose; 1F, 1-β-fructofuranosylnystose; S, Sucrose.



**FIGURE 4 |** Enzymatic kinetic of the selected fraction over aguamiel carried out at 70°C. K, Kestose; N, Nystose; 1F, 1-β-fructofuranosylnystose; S, Sucrose.

## Prebiotic Activity

Prebiotic activity showed different results between treated (T) and non-treated aguamiel (NT). The *Lactobacillus* species showed a faster growth over the aguamiel with a high concentration of FOS (T). In **Figure 5**, faster growth of both

*Lactobacillus* species can be observed, both species having their maximum growth between 12 and 16 h, compared with the same species evaluated over NT aguamiel (**Figure 6**) where *L. plantarum* 14917 did not grow and *L. paracasei* 25302 had its maximum growth at 24 h (almost half of the maximum growth of this bacteria in aguamiel with a high concentration of FOS). *B. bifidum* 450B showed a faster growth over the treated aguamiel, reaching its higher growth at 13 h, which was two times faster than the growth of  $2.1 \times 10^8$  cells/ mL over the non-treated aguamiel; in the non-treated aguamiel *B. bifidum* 450B reached its maximum growth of  $2.2 \times 10^8$  cells/mL at 24 h. *B. lactis* showed a higher growth over the non-treated aguamiel, the maximum growth of this bacteria was almost the double the maximum growth in the treated aguamiel.

## DISCUSSION

### Crude Enzymatic Extracts Evaluation

The evaluation of the extracts over aguamiel showed the influence of the carbon source used to induce the production of FOS producing enzymes. In the case of Orafit HSI<sup>®</sup>, these extracts showed hydrolytic activity, which increased as the fermentation progressed. Due to this behavior, the application of these extracts was discarded to the purpose of this work. Extracts obtained from aguamiel showed FOS production with extracts obtained at 24 and 30 h; this behavior was similar to the activities that Muñoz-Marquez et al. (17) reported, where the maximum fructosyltransferase activity was between 24 and 32 h of fermentation using *A. oryzae* DIA-MF. The extracts obtained from agavins fermentation showed FOS production at longer times (36 and 48 h) compared to that obtained from aguamiel, and the best FOS producer was the extract obtained at 48 h of fermentation at high-level agavins concentration. Other authors reported the production of the enzymatic extracts at the same times, Guio et al. (22) used *A. oryzae* N74 strain to produce FOS at 48 h of fermentation; Ottoni et al. (23) used *A. oryzae* IPT-301 strain to a final fermentation time of 72 h and Kurakake et al. (24) used *A. oryzae* KB at a final fermentation time of 96 h. Comparing the fermentation times to produce the enzymatic extract, the use of agavins or aguamiel to produce FOS producing enzymes at short fermentation times can be advantageous. Also, the concentration of the substrate has an important role in the expression of the enzymes; when comparing the FOS production of the extracts at the same time of fermentation from agavins, the production is over 10 times the production with the extracts from high agavins concentration.

### Fractioning and Enzymatic Activities

After the fractioning of the crude enzyme extract, different proteins were observed in the fractions (<30 and 30–100 kDa). The presence of inulinase activity in the <30 kDa fraction and the different proteins on the electrophoresis of this fraction compared to the 30–100 kDa fraction, suggest that one or more of these proteins may be responsible for this activity, but some researchers reported inulinases with low molecular weights for an inulinase of 30 kDa from *Rhizopus oligosporus* or 31 kDa from

**TABLE 3** | Antioxidant analysis of aguamiel without enzymatic treatment and aguamiel after the enzymatic treatment with the high FOS concentration.

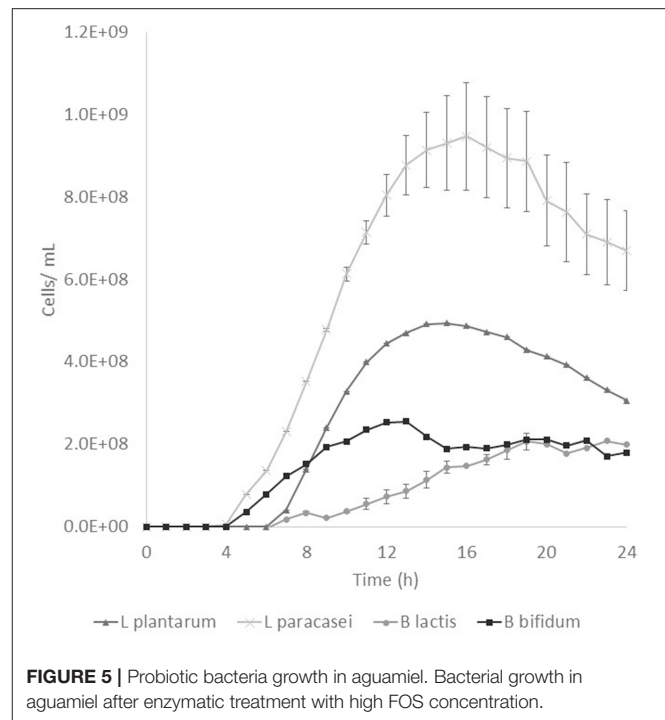
Sample	Antioxidant assay (mg/mg eq Trolox)	
	DPPH	FRAP
Aguamiel before enzymatic treatment	34.81 ± 5.75	42.65 ± 5.85
Aguamiel after enzymatic treatment	116.46 ± 0.48	298.86 ± 26.76

*Aspergillus ficuum* (25, 26). Also, the production of an inulinase with a lower molecular weight could be happening by *A. oryzae* DIA-MF induced by the agavins.

The higher hydrolytic and transfructosylating activity on the 30–100 kDa fraction can be produced by one or more of the proteins in it. If the responsibility of the enzymatic activity lies with one protein, then the enzyme could be a  $\beta$ -fructofuranosidase due to its high hydrolytic activity or it could be a conjunction of two enzymes; one  $\beta$ -fructofuranosidase and one fructosyltransferase with high hydrolytic and transfructosylating activity. Muñiz-Marquez et al. (17) reported the production of a fructosyltransferase with high transference activity produced by *A. oryzae* DIA-MF and Kurakake et al. (24) reported the production of two  $\beta$ -fructofuranosidases by *A. oryzae* KB, one with high transfructosylating activity and the other one with high hydrolytic activity. The production of only one enzyme with both activities or two different enzymes by the fungi could be possible. Spohner and Czermak (27) reported a fructosyltransferase with a molecular weight of 80 kDa approximately, Fernandes et al. (28) reported a  $\beta$ -fructofuranosidase with a molecular weight of 37 kDa similar to the Lincoln and More (29) report of a  $\beta$ -fructofuranosidase of 35 kDa, both from an *Aspergillus* species, and Kurakake et al. (24) reported the two  $\beta$ -fructofuranosidases of 96 and 79 kDa. The activity of more than one enzyme in the 30–100 kDa fraction with one or two activities could be occurring due to the presence of six different proteins.

## FOS Production

After the three temperature treatments in the enzymatic kinetic, the 70°C treatment did not show an increment in FOS and the 50°C showed an increment only at 2 h. This could be due to the low thermostability of the enzyme, at 70°C the proteins began to denaturalize immediately and at 50°C they were denaturalized but at a lower speed. The proteins could tolerate the 50°C for a short time but when applying longer times it begins to denaturalize; instead FOS production by enzymes with fructosylating activity showed their optimal temperatures between 50 and 60°C (8). According to this behavior in both treatments, we can conclude that the enzymes produced were not thermostable. The treatment of 30°C started producing kestose from the sucrose that aguamiel contains, as it is shown in **Figure 2**. The FOS production begins with kestose, and the

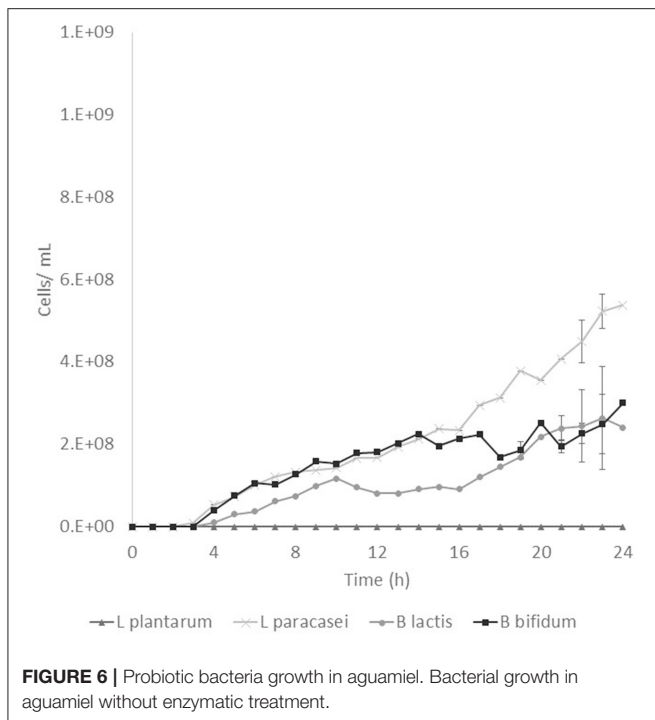
**FIGURE 5** | Probiotic bacteria growth in aguamiel. Bacterial growth in aguamiel after enzymatic treatment with high FOS concentration.

formation of nystose was next; this mechanism is the main mechanism that a transfructosylating enzyme uses to produce FOS from sucrose (8). In accordance with the mechanism of FOS formation, the formation of fructooligosaccharides with higher DP could take more time, the production of nystose is slower than kestose, and the concentration of kestose begins to decrease as it can be observed after 10 h of the enzymatic reaction, kestose is reduced in concentration as nystose continue increasing. The production of 1- $\beta$ -fructofuranosylnystose was not possible at the times the kinetic was carried out.

## Antioxidant Activity

FOS have been reported with antioxidant activity, Manosroi et al. (30) reported FOS extracted from *Coix lachryma-jobi* Linn to have an antioxidant activity similar to Vitamin C; Zhang et al. (31) evaluated the antioxidant capacity in fish fed with a low FOS concentration diet, and the results showed the increment of liver catalase and superoxide dismutase activities. The antioxidant activity was similar in both assays but not within treatments. Antioxidant activity from aguamiel after the enzymatic treatment was higher (116.46 ± 0.48 and 298.86 ± 26.76 mg/mg Eq Trolox for DPPH and FRAP, respectively), and in both cases the activity was 4 times better than the activity from the non-treated aguamiel (**Table 3**). Higher antioxidant activity could be related to the partial enzymatic degradation used to produce FOS or other fructose compounds could induce the formation of new antioxidants, such as heterocyclic compounds (5). Enzymatic hydrolysis of inulins and agavins could release FOS, increasing the content of terminal fructoses with reducing capacity able to participate in antioxidative reactions. Mesa et al. (32) also suggested that FOS with molecular masses lower or equal to 10





kDa are an important source of antioxidants that are able to scavenge peroxy radicals and to prevent *in vivo* LDL oxidation.

## Prebiotic Activity

FOS are compounds known as prebiotics due to their ability to reach the intestine microbiota and be used as a substrate by microbiota. It is known that *Lactobacillus* and *Bifidobacterium* are the two bacterial groups which can metabolize oligosaccharides, and both are part of the intestinal microbiota (4). The application of aguamiel with a high FOS concentration showed a different growth on the four bacteria used. A difference was observed in the growth pattern of the two *Lactobacillus* strains used. *L. plantarum* 14917 and *L. paracasei* 25302 had  $6.4 \times 10^7$  and  $9.1 \times 10^7$  cell/mL/h specific growth rates, respectively, in aguamiel after the enzymatic treatment. Meanwhile, the specific growth rate on the non-treated aguamiel was  $2.5 \times 10^7$  cell/mL/h for *L. paracasei* 25302; *L. plantarum* 14917 did not grow in this aguamiel. In addition, there was a significant statistical difference in the growth between the *Lactobacillus* strains. On the other hand, *Bifidobacterium* bacteria growth has similar behavior, the specific growth rate of *B. lactis* and *B. bifidum* 450B in the treated aguamiel was  $1.5 \times 10^7$  and  $3.1 \times 10^7$  cell/mL/h, respectively, compared to the growth on the non-treated aguamiel which was  $1.3 \times 10^7$  and  $5.3 \times 10^6$  cell/mL/h. *B. lactis* had similar

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growth on both aguamiel, whereas, *B. bifidum* 450B had the same behavior of the *Lactobacillus* strains, with a higher specific growth rate. Higher growth of probiotic bacteria could be related to the production of  $\beta$ -fructofuranosidase, and its participation in conjunction with the sucrose phosphoenolpyruvate transport system to consume the substrate and provide the enzyme with a better substrate consumption for its growth (33). Due the high sucrose concentration in the non-treated aguamiel, this could be affecting the bacterial growth. The treated aguamiel demonstrated better conditions for improving the probiotic bacterial growth and due to the capacity of FOS to reach the intestinal microbiota, the potential for use of aguamiel as a food rich with prebiotic compounds is high.

Finally, we can conclude that aguamiel treated with the enzymatic fraction showed a high conversion of sucrose to FOS. This conversion indicates an opportunity to use aguamiel as a functional food due to the concentration of FOS, which was increased from  $1.61 \pm 0.08$  to  $31.01 \pm 3.42$  g/L after 10 h reaction at  $30^\circ\text{C}$  applying 10% enzymatic fraction-substrate (v/v). Considering the minimum daily effective intake of FOS, a portion of 100 mL of treated aguamiel could promote health benefits, compared to a portion of  $\sim 2$  L of non-treated aguamiel. Antioxidant activity was highly increased (34.81–116.46 mg/eq Trolox in DPPH assay and 42.65–298.86 mg/eq Trolox in FRAP assay) and growth of probiotic bacteria was higher in aguamiel after the enzymatic treatment. Due to the FOS concentration in the treated aguamiel, this product proved to have higher antioxidant activity and probiotic bacterial growth. After the application of the enzymatic treatment, aguamiel was enriched with FOS which improved antioxidant and prebiotic properties, therefore, it can be used as a functional food.

## AUTHOR CONTRIBUTIONS

BP executed all the experiments presented in this paper, within the framework of his postgraduate thesis. AF-G conducted the experiments that were presented and supervised the work performed by BP, as his thesis director. AI advised the experiments related to the fractionation of proteins and facilitated the materials to be able to carry it out. RR-J advised BP's work regarding the quantification of sugars present in aguamiel. CA participated in the co-direction of the work and in conjunction with AF-G and BP, designed the experiments and supervised the execution of the same.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Microbial Pigments in the Food Industry—Challenges and the Way Forward

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Developing new colors for the food industry is challenging, as colorants need to be compatible with a food flavors, safety, and nutritional value, and which ultimately have a minimal impact on the price of the product. In addition, food colorants should preferably be natural rather than synthetic compounds. Micro-organisms already produce industrially useful natural colorants such as carotenoids and anthocyanins. Microbial food colorants can be produced at scale at relatively low costs. This review highlights the significance of color in the food industry, why there is a need to shift to natural food colors compared to synthetic ones and how using microbial pigments as food colorants, instead of colors from other natural sources, is a preferable option. We also summarize the microbial derived food colorants currently used and discuss their classification based on their chemical structure. Finally, we discuss the challenges faced by the use and development of food grade microbial pigments and how to deal with these challenges, using advanced techniques including metabolic engineering and nanotechnology.

**Keywords:** microbial pigments, natural colorants, *Monascus* pigments, metabolic engineering, microencapsulation, food color

## INTRODUCTION

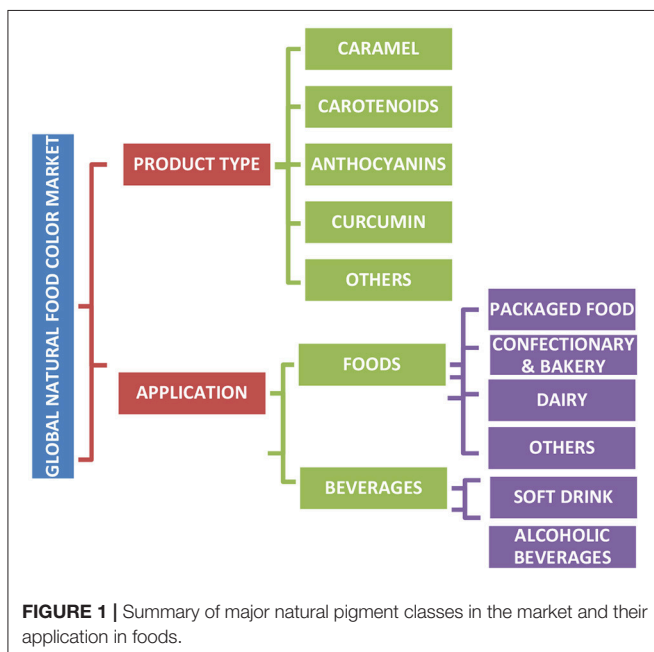
Color plays a significant role in the food production and processing sector, contributing to the sensory attribute of food. It signifies freshness, nutritional value, safety, and aesthetic value of a food, directly affecting the market value of the colored food product (1–3). Food coloring is presumed to have originated back in 1500 BCE (4). Ancient Roman and Egyptians writings show activities such as the coloring of drugs and wine. In earlier times, most of the food coloring agents were derived from natural sources such as paprika, berries, turmeric, indigo, saffron, and various flowers (5, 6). In the 1800's there was a shift toward development of synthetic colors due to their chemical stability, low production cost, and larger ranges of hue and shade. The first synthetic dye, Perkin's Mauve pigment, appeared in 1856 (4), which also lead to the discovery of other synthetic dyes. However, possible side effects of synthetic colors like hyper-activity in children, allergenicity, toxicological, and carcinogenicity problems, has led to the banning of many synthetic food colorants further leading to a transition from the use of synthetic food colors, to natural ones (7–9). An increase in the desire to label food as natural has also contributed to a decline in the use of synthetic food colorants.

Research on natural food colors has become a key area in the food industry, particularly the discovery of new natural colorants. The use of compounds as food colorants is highly regulated, whether the colors are naturally derived or synthetically produced. Organizations such as the United States Food and Drug Administration (FDA), the European Food Standards Authority (EFSA), and The World Health Organization (WHO) have advocated safe dosages for the use of these colors in food, drugs, and cosmetic items (9–11).

Food colorants exempt from certification generally include natural pigments, but no legal definition for the term ‘natural’ has been adopted yet, leading to consumer, and industrial confusion. Colorants exempted from certification include a variety of pigments obtained from microbial, plant, mineral and animal sources but also include synthesized compounds that are identical to natural products, despite the common belief that colorants exempt from certification are all natural (12).

Natural colors are assumed safe if they are non-allergic, non-toxic, non-carcinogenic, and biodegradable, thereby rendering no risk to the environment (5, 10). Due to the lower risk advantage of natural colors and changing perceptions of consumers to consume natural products, there is an increasing interest in the discovery of new natural colors. The consumer demand for natural colors and their growth as a category is predicted to increase by 7% annually (13–15). In recent times, natural food colors have varied applications in the food industry, with almost all major natural pigment classes being used in at least one sector of the food industry (Figure 1).

Despite the benefits, that come with using natural colors, these pigments often have drawbacks compared with synthetic colors. In many cases, potential natural pigments that can be used as food colorants present many challenges such as higher cost and lower stability.



Natural colors are primarily derived from plants, insects, mineral ores or microbial sources. Microbial colorants are preferable because of scalability ease as well as a potentially lower cost of production (4, 11). Microbial fermentation for the production of natural pigments have several benefits such as cheaper production, higher yields, easier extraction, lower-cost raw materials, no seasonal variations, and strain improvement techniques to increase natural pigment (16). These can also have health benefits like anticancer activity, antimicrobial activity and antioxidant activity (1, 17). Microbes produce a variety of pigments that can be used as food colors such as carotenoids, flavins, melanins, quinines, monascins, violacein, amongst others. They can also be used as additives, antioxidants, color intensifiers, and functional food ingredients (3, 18).

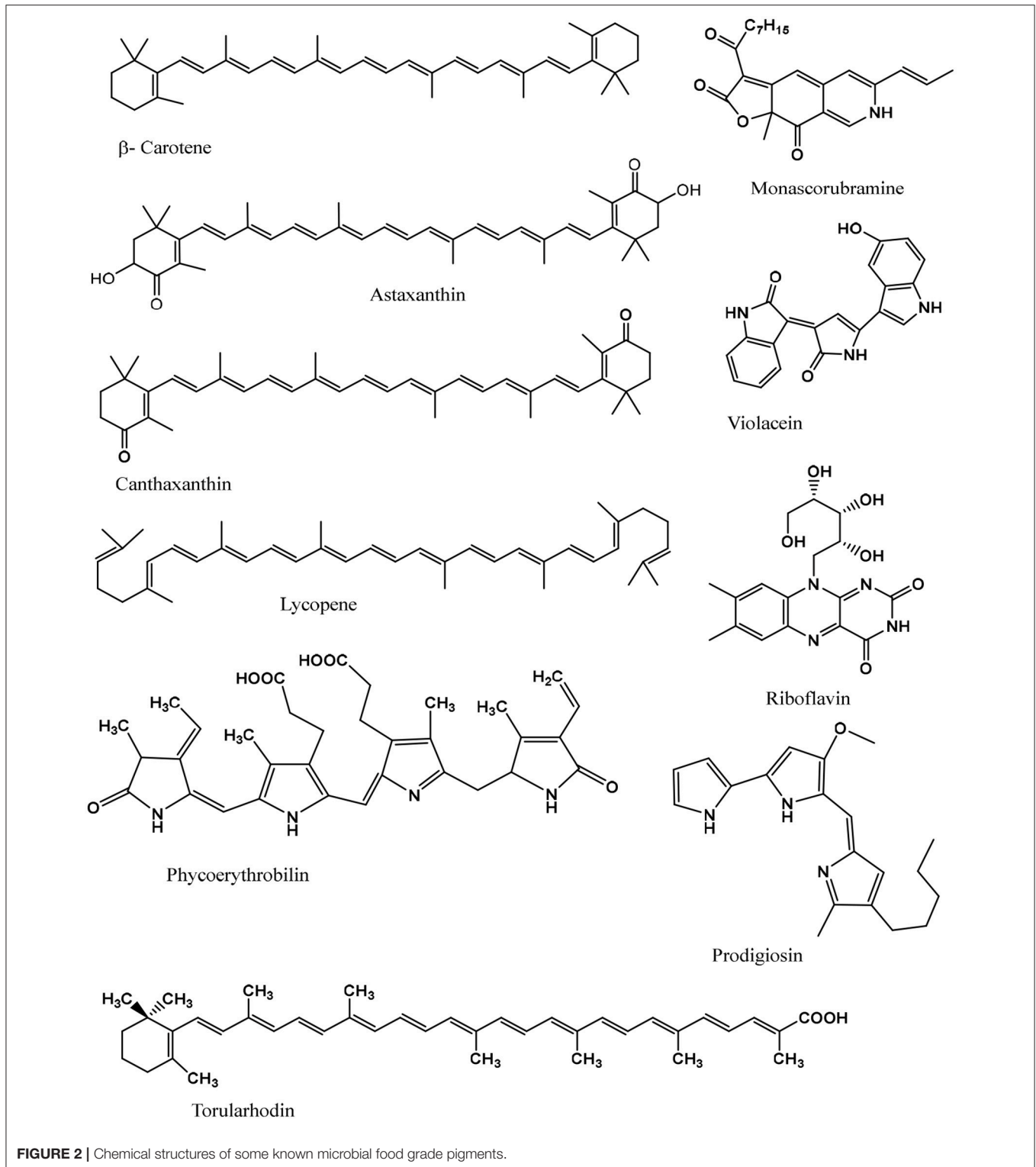
Advances in organic chemistry and metabolic engineering have enabled the mass production of microbes of interest. Studying the biosynthetic pathway for pigment production can help in understanding the roadblocks in the production of pigments and to counter that, genes can be cloned, and recombinant DNA technology can be used to increase pigment production (19, 20). Using the appropriate fermentation strategies and modifying conditions to be more suitable for the production of pigments, developing low cost processes and extraction processes, co-pigmentation strategies, have all been applied for efficient microbial pigment production. Newly emerging tools such as nanotechnology has also been effectively used in the food industry, including in pigment formulation (21). Nanotized natural food colorants derived from microbial sources can increase stability, shelf life, or solubility, leading to better delivery systems for food, and feed (22). The present review focuses on the potential of microbial pigments used as food colorants, their benefits and challenges; explores possible strategies for simplifying the process for overproduction of pigments in microbial systems, as well as the methods to improve pigment stability and formulation.

## MICROBIAL PIGMENTS THAT CAN BE USED AS FOOD GRADE COLORS

Some of the major pigments found in micro-organisms which are used as food colorants are canthaxanthin, astaxanthin, prodigiosin, phycocyanin, violacein, riboflavin, beta-carotene, melanin, and lycopene, shown in Figure 2 and a more extensive list given in Table 1. Microbial pigments can be either inorganic or organic, although organic pigments tend to be more useful as food colorants.

- i. **Canthaxanthin**- is an orange to deep pink colored carotenoid that is lipid soluble and a potent antioxidant. It is isolated from *Bradyrhizobium* Sepp, is a trans-carotenoid pigment, and is approved as a food colorant and used in a range of foods as well as salmon and poultry feed (100–102).
- ii. **Astaxanthin**- is a red-orange pigment, naturally found in basidiomycetous yeast, microalgae, salmon and crustaceans, red shrimp, cray fish, feathers of some birds, and is lipid





soluble (35, 37, 103–105). It's an approved coloring agent used in fish and animal foods (106).

iii. **Prodigiosin**- Many strains of *Serratia marcescens*, produce a red pigment, which shows antibacterial, antimalarial, antibiotic and antineoplastic activity (34, 70, 107). It has been

successfully applied as coloring agents in yogurt, milk and carbonated drinks (108).

iv. **Phycocyanin**- is a blue pigment produced by chlorophyll A containing cyanobacteria. *Aphanizomenon flos-aquae* and *Spirulina* produces phycocyanin which is being used in the

**TABLE 1** | Microbial pigments that are being used or with high potential to be used as natural food colorants.

Sr. No.	Pigment	Color	Microorganism	Bioactivity	Status*	References
<b>ALGAE AND MICROALGAE</b>						
1	Astaxanthin	Pink-red	<i>Haematococcus pluvialis</i> Microalgae	Antioxidant photo protectant, Anticancer, Anti-inflammatory	IP	(23–26)
2	β- carotene	Orange	<i>Dunaliella salina</i> Microalgae	Anticancer, Antioxidant suppression of cholesterol synthesis	IP	(26–29)
3	Lutein	Yellow	<i>Chlorella and others</i> Microalgae	Antioxidant	IP	(26, 30–32)
4	Phycocerythrin	Red	<i>Porphyridium cruentum and many other microalgae and cyanobacteria</i> Algae, Cyanobacteria	Antioxidant, Antitumor activity, Immunoregulatory	DS	(26, 33)
5	Phycocyanin	Blue	<i>Arthrospira sp. (formerly Spirulina sp.) and many other microalgae and cyanobacteria</i> Algae, Cyanobacteria	Antioxidant, Antitumor, Immunoregulatory	IP	(26, 34–37)
<b>ARCHAEA</b>						
6	Canthaxanthin	Orange	<i>Haloferax alexandrinus</i> Archaea	Antioxidant, photoprotectant, Anticancer, Anti-inflammatory	NK	(38–41)
<b>BACTERIA</b>						
7	Astaxanthin	Pink-red	<i>Agrobacterium aurantiacum</i> Bacteria <i>Paracoccus carotinifaciens</i> Bacteria	Antioxidant Anticancer Anti-inflammatory, Antioxidant Anticancer	RP/IP	(23, 42)
8	Canthaxanthin	Orange	<i>Bradyrhizobium</i> spp. <i>Lactobacillus pluvialis</i> .	Antioxidant, Anticancer	RP	(38–41)
9	Granadaene	Orange-red	<i>Streptococcus agalactiae</i>	Antioxidant, detoxify ROS	DS	(43, 44)
10	Heptyl prodigiosin	Red	α-Proteobacteria	Antiplasmodial	DS	(45)
11	Prodigiosin	Red	<i>Serratia marcescens</i> <i>Pseudoalteromonas rubra</i>	Anticancer, DNA Cleavage, Immunosuppressant	IP	(46–49)
12	Phycocyanin	Blue, green	<i>Pseudomonas</i> spp.	Cytotoxicity, Neutrophil apoptosis, Ciliary dysmotility, Proinflammatory	IP	(50)
13	Rubrolone	Red	<i>Streptomyces echinoruber</i>	Antimicrobial	DS	(51–53)
14	Staphyloxanthin	Golden	<i>Staphylococcus aureus</i>	Antioxidant, detoxify ROS	NK	(54–56)
15	Trypitanthin	Light-dark Yellow	<i>Cytophaga/Flexibacteria AM13,1 Strain</i>	Antioxidant, Anticancer	NK	(57)
16	Undecylprodigiosin	Red	<i>Streptomyces</i> sp.	Antibacterial, Antioxidative, UV-protective, Anticancer	NK	(54, 55, 58, 59)
17	Violacein	Purple	<i>Janthinobacterium lividum</i> , <i>Pseudoalteromonas tunicate</i> , <i>Pseudoalteromonas</i> spp. <i>Chromobacterium violaceum</i>	Antioxidant, detoxify ROS	NK	(60–62)
18	Zeaxanthin	Yellow	<i>Staphylococcus aureus</i> , <i>Flavobacterium</i> spp., <i>Paracoccus zeaxanthinifaciens</i> , <i>Sphingobacterium multivorum</i> Bacteria	Photoprotectant, Antioxidant	DS	(63)
<b>CYANOBACTERIA</b>						
19	Scytonemin	Reddish Brown	<i>Cyanobacteria</i> Cyanobacteria	Anti-inflammatory, Antiproliferative	NK	(64)
<b>FUNGI</b>						
20	Ankaflavin	Yellow	<i>Monascus</i> sp.	Antitumor, Anti-inflammatory	IP	(65, 66)
21	Anthraquinones	Red and other hues Known as Arpink red or Natural Red	<i>Penicillium oxalicum (and many other fungi)</i>	Antifungal, Virucidal	IP	(67–69)

(Continued)

TABLE 1 | Continued

Sr. No.	Pigment	Color	Microorganism	Bioactivity	Status	References
22	Azaphilones	Red	<i>Talaromyces atroroseus</i> <i>Penicillium purpurogenum</i>	Antioxidant, Anticancer Antioxidant	DS	(70–75)
23	$\beta$ - carotene	Yellow-orange	<i>Blakeslea trispora</i> , <i>Fusarium sporotrichioides</i> , <i>Mucor, circinelloides</i> , <i>Neurospora crassa</i> , <i>Phycomyces</i> , <i>Blakesleeanus</i>	Anticancer, Antioxidant, suppression of cholesterol synthesis	IP	(24, 35, 66, 76–78)
24	Canthaxanthin	Orange, pink	<i>Monascus</i> spp.	Antioxidant, Anticancer	NK	(34, 35, 39, 40, 79)
25	Cycloprodigiosin	Red	<i>Pseudoalteromonas denitrificans</i>	Antiplasmodial, Anticancer	DS	(80, 81)
26	Lycopene	Red	<i>Fusarium Sporotrichioides</i> , <i>Blakeslea trispora</i>	Antioxidant, Anticancer	RP/DS	(82, 83)
27	Monascorubramin	Red	<i>Monascus</i> spp.	Antioxidant, Anticancer	IP	(34, 84)
28	Naphtoquinone	Deep blood red	<i>Cordyceps unilateralis</i>	Anticancer, Antibacterial, Trypanocidal	RP	(70, 85–87)
29	Riboflavin	Yellow	<i>Ashbya gossypii</i>	Anticancer, Antioxidant, protection against cardiovascular diseases, in vision	IP	(34, 88–90)
30	Rubropunctatin	Orange	<i>Monascus</i> spp.	Anticancer	IP	(84, 91)
31	Xanthomonadin	Yellow	<i>Xanthomonas oryzae</i>	protection against photo damage	NK	(35, 92)
<b>YEAST</b>						
32	Astaxanthin	Pink-red	<i>Xanthophyllomyces dendrorhous</i> formerly <i>Phaffia rhodozyma</i>	Antioxidant, photoprotectant, Anticancer, Anti-inflammatory	DS	(93–95)
33	Melanin	Black	<i>Saccharomyces</i> , <i>Neoformans</i>	Antimicrobial, Antibiofilm and antioxidant	NK	(96)
34	Torularhodin	Orange-red	<i>Rhodotorula</i> spp.	Antioxidant, Antimicrobial	DS	(97–99)

\*Industrial status adopted from Dufossé. (34, 42, 66).

DS, Development stage; IP, Industrial production; RP, Research project; NK, Not Known.

food and beverage industry as the natural coloring agent ‘Lina Blue’ and is also found in sweets and ice cream (36, 109, 110).

- v. **Violacein**- *Chromobacterium violaceum* is one of the most prominent producers of this purple pigment, other bacterial species also produces the pigment and mostly have a purple hue. It exhibits antifungal, antibiotic, antitumor and antibacterial properties. Violacein has shown potential use in food, cosmetic and textile industries (34, 60–62).
- vi. **Riboflavin**- Water soluble vitamin B2, is a yellow colored pigment and produced by various microorganisms. It is used in diary items, breakfast cereals, baby foods, sauces, fruit drinks, and energy drinks (34, 88–90).
- vii. **Beta-carotene**- A red-orange colored organic pigment, mostly extracted from the beta-carotene rich algae, *Dunaliella salina* (111). Production of  $\beta$ -carotene through fermentation of *Blakeslea trispora* produces a pigment equivalent to pigments produced through a chemical process and is an acceptable coloring agent (24, 35, 66, 76–78). It is used in a variety of food items ranging from red to yellow in color.
- viii. **Melanin**- Melanins are natural pigments present in animals, plants and in many micro-organisms. They are widely used in eye glasses, cosmetic, food items, sunscreen protection creams, pharmaceuticals and food items (35, 70, 96, 109, 112, 113).

- ix. **Lycopene**- widely present and consumed in tomatoes, a brilliant red pigment consisting of carotenoid. It has been isolated from microbes like *Fusarium*, *Sporotrichioides*, and *Blakeslea trispora*, and has the potential to attenuate persistent diseases such as some types of cancers and coronary heart disease (82, 83). It is used in meat coloring in countries like the USA, Australia and New Zealand.

## THE BENEFITS OF USING MICROBIAL PIGMENTS AS FOOD GRADE COLORING AGENTS

Micro-organisms are found in almost every environmental niche and have various roles in nature. They are also affiliated with food and are accountable for the fermentation of food products. Microbial pigments are a better alternative to synthetic food colors compared to plants because of their availability, non-seasonality, scalability, higher yield per hectare, and straight forward down streaming processing. Microbial pigments like that of *Monascus*, Arpink Red (natural red- industrial name) from *Penicillium oxalicum*,  $\beta$ -carotene from *Blakeslea trispora* and Astaxanthin from various microbes are already used in the food industry to color foods (34, 113, 114). A lot of research has been done to lower production and processing costs for natural colors,

to increase stability and shelf life, so that it can compete with the use of synthetic colors. Many of these pigments not only work as coloring agents but also impart health benefits (Bioactivity of various microbial pigments mentioned in **Table 1**). Microorganisms produce an large quantities of pharmacologically and biologically active compounds that can have a diverse range of activities, including antioxidants, antimicrobial, anticancer, immuno-regulatory, and anti-inflammatory compounds.

### Antioxidant Activity

Microbial pigments like violacein, carotenoids, anthocyanins, and naphthoquinone have been shown to be potent antioxidants agents. Violacein which is a purple pigment largely produced by *Pseudoalteromonas* and *Chromobacter violaceum* (60, 62) is a powerful antioxidant which stimulates mucosal defense mechanisms to protect against oxidative damage in gastric ulcers (115, 116). *Staphylococcus aureus* produces a yellow pigment called staphyloxanthin, that prevents carbon tetrachloride induced oxidative stress in swiss albino mice (117). There are many other pigments that can act as antioxidants such as Astaxanthin, Granadaene, Canthaxanthin, Lycopene, Riboflavin,  $\beta$ -carotene, Torularhodin, etc.

### Anticancer Property

Anticancer activities in microbial pigments have been reported in a number of studies. These pigments can induce apoptosis, which lead to the destruction of cancerous cells. Scytonemin which is a green-yellow pigment, produced by the aquatic cyanobacteria, inhibits the action of the cell cycle regulatory protein kinase, thereby showing an antiproliferative effect (64). Prodigiosin is red pigment which is a potent anticancer compound, produced by *Serratia marcescens* and *Pseudomonas rubra*. It shows an apoptotic effect against human cervical carcinoma (118). Anticancer activity is shown by synthetic indole derivatives and analogs of prodigiosin *in-vitro* (119). Violacein showed cytotoxic effects on HL60 leukemia cells through a TNF signaling cascade and the activation of Caspase-8 and p38 MAPK (120). There are various pigments that can act as anticancer agents such as Astaxanthin, Canthaxanthin, Lycopene, Monascorubramin, Riboflavin, Rubropunctatin,  $\beta$ -carotene, Torularhodin, and others.

### Antimicrobial Activity

Many micro-organisms produce antimicrobial compounds, some of which are presently used as antibiotics. A pigment obtained from an endophytic fungus was shown to be more potent than the commercially available antibiotic Streptomycin. It was effective against bacteria like *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi* and *Vibrio cholera* (121). It is known that violacein causes growth inhibition, additionally also killing the bacteria. It also exhibits antifungal, antiprotozoal and antiviral activities (76, 77, 122). The recent emergence of antibiotic and multi drug resistant microbial strains has led to a search for new and novel compounds that can be used as antibiotics. Finding novel microbial pigments that have both pigment producing and antimicrobial properties is highly advantageous (123).

## CHALLENGES FACED IN NATURAL FOOD COLORS

Even though there are many types of natural pigments from various microbial sources, the commercial development of natural pigments as food colorants is challenging. Regulatory hurdles are high for the development of any new compounds for food use, including as a colorant. The cost of using natural colors is five times more than using synthetic colors, especially when used in confectionary items, where it can be 20 times more expensive (124). Substantial quantities of raw materials are required to produce equal quantities of natural colors than synthetic colors. Higher dosages of a natural color are normally needed for the desired hue, thereby increasing the cost.

Natural pigments have many product challenges with respect to cost, application, process, and quality. Microbial pigments have a weaker tinctorial strength and may react on different food matrices, causing undesirable flavors and odors. Synthetic food colorants that the food industry came to rely on over the past 50–60 years are relatively well-behaved and consistent in their performance. Replacement of synthetic colors with natural colors in the food industry is challenging, particularly with regard to the relatively low range of natural colors approved for food use. Deodorization is another issue that arises in natural pigment products as many of the available natural pigments have an odor that is undesired in the food products. Furthermore, natural colors are generally more sensitive to light, pH, UV, temperature, oxygen, and heat, leading to color loss caused by fading and a decreased shelf life. Some natural pigments are sensitive to other ambient conditions like metal ions, proteins or organic compounds (10, 125, 126). It is well-known that vitamin C will enhance the stability of beverage products, which are colored with carotenoids like beta-carotene and paprika oleoresin, but the same vitamin will cause the degradation of anthocyanins (127).

Major microbial pigments like carotenoids, chlorophyll, anthocyanins, and others also face such limitations. Carotenoids, which are strongly colored isoprenoid plant compounds and highly conjugated, are unstable when exposed to oxygen or light (128–130). Chlorophyll undergoes rapid degradation due to enzymatic reactions or factors like light, oxygen, heat or acid, leading to the formation of chlorophyll derivatives (131). Formulation of these natural colors is challenging and methods such as micro-encapsulation can be applied to improve stability and in some cases solubility. Many fungal pigments are prohibited as natural colorants due to the presence of mycotoxins (132). It is therefore important to use non-toxic and non-pathogenic strains for natural pigment extraction. When a promising pigment-producing microbe is discovered, metabolic engineering can be used for controlled biosynthesis of the pigment and toxin production.

## TECHNOLOGIES FOR ENHANCING PIGMENT PRODUCTION

The idea is to bring microbial pigments out of petri plates and on to the market (3, 34). There is a need to find alternative colorants



that are cost effective, completely natural, non-toxic and which do not produce any recalcitrant intermediates. Commercial success of a natural pigment is dependent on the investment made to obtain the final product, its regulatory approval and its influence in the market. Three key operations are important in the industrial production of natural pigments: Discovery of newer and novel alternative sources; cost effective production with uniform quality; and improved applicability (133). Rigorous trials are required to develop methods that stabilize natural pigments in different food matrices, increase shelf life, prevent the influence of various environmental parameters on the pigment, finding inexpensive organic substrates for the growth of the pigment producing micro-organisms and making the fermentation process more cost effective (134).

## Newly Developed Smarter Screening Methods

There are many new advances in the quick and easy detection of microbial pigments. One of the best examples is the condensed handheld Raman spectrometer, used for detecting pigments with the help of a 532 nm excitation laser. This can detect common and uncommon carotenoids, bacterioruberin and other known pigment compounds. This hand-held device has been used to identify microbial pigments in various ecological niches including halophilic micro-organisms (135, 136).

Intelligent screening also includes having prior knowledge of the toxic metabolite pathway of the pigment producer, so that toxic and pathogenic pigment producers can be ruled out or manipulated for food coloring purposes. *Fusarium venenatum* produces a mycelial food product Quorn™, which is also known to produce a cytotoxic compound called 4, 15-diacetoxyscirpenol (137).

Mass spectrometry with electrospray ionization can also be used for faster identification of pigment producing fungal strains and for grouping them in classes and subclasses (138). More than 15,000 microbial metabolites are already known and so rapid dereplication and identification of known compounds is important. HPLC, mass spectrometry, LCMS, nuclear magnetic resonance (NMR), and UV-VIS spectra can be applied to the rapid identification of known compounds even within relatively complex mixtures, without the need for individual compounds purification (139).

## Strain Development and Fermentation

There are several challenges linked to scaling up the production of microbial pigments, but recent advances in technology has helped in somewhat overcoming these challenges. The use of fermentation tanks for large scale production of pigments, the use of strain improving techniques and strain development through random mutagenesis and multiple selection rounds has helped to develop a cost effective and industrially viable production process for pigments and other natural compounds. Strain development is important because the pigments produced by wild type strains are often too low in quantity and take longer fermentation times, making the process uneconomical. Strain improvement is done by common mutagens like 1-methyl-3-nitro-1-nitrosoguanidine (NTG), Ethyl methyl sulfonate (EMS) and Ultraviolet

(UV), which can lead to a several-fold increase in pigment production (140–142).

Medium optimization is an important process for maximizing yield of the fermentative product. Optimizing the medium includes controlling operating conditions like temperature, pH, aeration, agitation, and media components. Response surface methodology (RSM) is an effective approach for the process optimization of pigment production. This solves the multivariate data obtained to solve multivariate equations, thereby reducing the number of experimental trials needed to evaluate multiple variables (142, 143). Su et al. developed an optimal medium composition, which can be used for culturing *Serratia marcescens* in the production of prodigiosin. Sucrose and glycine were added as a carbohydrate and energy source, which increased the production of prodigiosin by 2.12–2.15 folds. Inorganic supplementation with KH<sub>2</sub>PO<sub>4</sub> accelerated cell growth, leading to the increased production of prodigiosin (144). To develop an economical production process, efficient fermentation design and standardization of the medium is important. Application of statistical techniques can result in an improved output response and can reduce variability and overall costs (21).

## Cost-Effective Downstreaming

Developing more cost-effective recovery and separation techniques for microbial pigments are also needed. Large-scale separation and recovery of pigments using conventional methods is costly. Extraction using organic solvents is a complicated and time-consuming process, in which substantial amounts of organic solvents are exhausted while the yield of the high purity product can be extremely low. In addition, using solvents other than water and ethanol can defeat the purpose of obtaining a natural pigment for regulatory purposes, since most organic solvents are not natural. The technique of using non-ionic adsorption resins for an efficient separation and purification has been applied to many nucleic acids, organic acids, peptides, and others (145, 146). These resins have a high loading ability, thereby helping in recovering of compounds in large quantities. In addition, these resins can directly be used to adsorb compounds from the culture broth. It helps in lowering the cost of separation, by lessening the consumption of extraction solvents and increasing its reusability. An efficient method for prodigiosin separation and purification was described by Wang et al. who used non-ionic resins directly from the culture broth, thereby eliminating the cell separation step, yielding a concentrated and semi-purified product (147).

## Metabolic Engineering

Recent developments in molecular biology and metabolic engineering have led to the cloning of genes responsible for pigment biosynthesis and enabled overproduction of these pigments by gene manipulations. Pigment biosynthetic pathways have been extensively studied and engineered to overproduce a pigment and to change the pigments' molecular structure and color. Blue pigment Actinorhodin, produced by *Streptomyces coelicolor*, has been genetically manipulated to produce a related bright yellow polyketide known as kalafungin, that is used to produce an anthraquinone, which is a reddish-yellow color

(148, 149). Heterologous expression has been used to develop cell factories to efficiently produce pigments by expressing biosynthetic pathways from novel or known pigment producers (150, 151).

Understanding the biosynthetic pathways for microbial pigments is an extremely important starting point, followed by identifying genes and the gene cascades responsible for pigment production, then engineering these genes for over production. Cloning the genes responsible for pigment biosynthesis into microbial vectors, like bacterial or yeast cells, has become a cost-effective and more economical industrial production process. Industrially reliable micro-organisms such as *E.coli*, *Bacillus subtilis*, *Pseudomonas putida*, *Corynebacterium glutamicum*, and *Pichia pastoris*, can be used to develop of tailor-made recombinants, genetically engineering the production of pigments (152).

Techniques like selected and random mutagenesis are used to obtain hyper-producing strains, and for this, chemicals and physical methods such as 1-methyl-3-nitroguanidine, antimycin A, or Ethyl methane sulfonate and Gamma radiation and UV light are employed (153). Carotenogenic genes from *Xanthophyllomyces dendrorhous* or *Erwinia uredovora* or *Agrobacterium aurantiacum*, yeasts like *Candida utilis* and *Saccharomyces cerevisiae* are genetically manipulated to produce carotenoids like lycopene or  $\beta$ -carotene or astaxanthin (154–156). At this time, engineering genes responsible for carotenoid pigments, has been limited to non-carotenogenic micro-organisms like *C.utilis* or *S. cerevisiae*. There is almost no published data on the metabolic engineering of wild type carotenoid producers like *Dunaliella salina*, *B. trispora* and *R. mucilaginosa*. Wang et al. (157) used metabolic engineering and mutagenesis to enhance carotenoid production in *R.mucilaginosa* KC8, which produces carotenoids, mainly  $\beta$ -carotene and torularhodin (157). Grewal et al. (158) described betaine production in a heterologous microbial host *Saccharomyces cerevisiae*, using glucose as a substrate. They also established that novel betalain derivatives could be obtained by feeding different amines in the culture (158).

In the case of *Monascus*, three polyketide pigments are produced namely Citrinin, red pigments and monacolin K (159, 160). Various techniques have been tried to decrease the production of citrinin, a mycotoxin, and to increase the production of the red pigment. Changes in the nitrogen composition, dissolved oxygen, pH, and genetic alterations are some of the various techniques tried to minimize citrinin. The polyketide synthase gene responsible for biosynthesis of citrinin has been studied in *Monascus purpureus*. In the industrial strain *M. purpureus* SM001, the polyketide synthase gene pksCT has been successfully cloned to eliminate citrinin production (161–163).

## METABOLIC ENGINEERING USING THE CRISPR-CAS9 SYSTEM

CRISPER-Cas9 has created a trend and various laboratories are using the technology for newer applications in biology, especially

genome engineering. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. It consists of two key components that brings about the change in DNA, the first being the enzyme Cas9, which acts like molecular scissors and makes double stranded cuts at the target location, helping in adding, or removing pieces of DNA. The second component is a piece of RNA, also known as the guide RNA, which is a pre-designed sequence of about 20 bases, and which is located inside a longer RNA scaffold. This scaffold binds to the target DNA sequences and the guide RNAs directs the Cas9 enzyme to make cuts at the right point in the genome. Due to the cuts being made, the cell activates its DNA repair machinery and tries to repair the damages, which can be efficiently used for introducing changes to one or more genes in the genome (164). Hence CRISPER-Cas 9 system can be very well used for metabolic engineering in bacteria, yeast and fungi to make them cellular factories for cost efficient production of natural food colors (165).

CRISPER- Cas9 has been efficiently used in the production of industrially important metabolite compounds. It can be used in a wide variety of bacterial cells such as *Corynebacterium*, *Escherichia coli*, *Pseudomonas*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Lactobacillus*, *Mycobacterium* and *Streptomyces*, genetically modifying them to produce metabolites such as biofuel, biochemical, pharmaceutical precursors, or any other significant metabolite (166). The CRISPER system has been used in the industrial yeast *Saccharomyces cerevisiae*, as it is one of the most noticeable cell factories for the industrial production of a large number of products. It can also be engineered to produce natural colors, if a color-producing gene is inserted into its genome using the CRISPER-Cas9 system (167).

Metabolic engineering in filamentous fungi have been extremely tough due to various reasons such as a lack of genetic markers and even when they are available, it remains a tedious process because of low gene-targeting frequencies. The CRISPER-Cas9 system has been employed in *Neurospora crassa* (168), *Aspergillus nidulans* (169) and in several other species of filamentous fungi such as *Magnaporthe oryzae* (170) and *Trichoderma reesei* (171). Nielson et al. have developed a system for *Aspergillus nidulans*, harboring the CRISPER-Cas 9 system that can potentially be applied in many fungal systems with close to no adaptation. They showed that it was useful in an extensive array of filamentous fungi (151). They even used the same system in *Talaromyces atrovirens*, which is a major natural red color producer in the food industry. Recently, the CRISPER-Cas9 system was used in *Penicillium chrysogenum* (172) showing a rapid improvement of engineering filamentous fungi. Limited research studies on using CRISPER-Cas9 in micro-organisms for pigment production exist. More research is required to optimize the use of the CRISPER system for this application.

## ADDRESSING INSTABILITY OF NATURAL PIGMENTS

To be industrially useful, microbial pigments need to be stable against environmental factors like light, pH, temperature, UV, and food matrices. Many microbial pigments are rendered useless

because of their instability against ambient conditions and have short shelf life. There are various techniques available that can produce a more stable natural pigment, which has a higher shelf life and market value in terms of the cost-effective stability measures taken.

## Microencapsulation, Nanoemulsions, and the Formation of Nanoformulations

Micro-encapsulation and nano-formulations can be applied to stabilize, improve solubility and deliver natural pigments to food matrices. Natural colors like anthocyanins and carotenoids, have stability issues in various environmental conditions and also present solubility problems in some matrices (173). Micro-encapsulation can be defined as packing any solid, gas or liquid in sealed capsules of sizes ranging from millimeters to nanometers (174). The core or the active compound becomes the packaging material, in this case the microbial pigment and the packaging material, is called the wall or shell material (174). The wall material used should have emulsifying properties, low viscosity, be biodegradable, should have film forming properties, should resist GIT, be low cost and should show low hygroscopicity (175). There are various wall materials that are currently used to encapsulate microbial pigments for use as food color such as maltodextrins, modified starch, inulin, fucellaran and others (176).

Encapsulated colors are easier to handle, have better solubility, and show improved stability to ambient conditions, leading to an increased shelf life. The wall material protects the active core material from light, temperature, oxygen, humidity, and matrix interactions. The major objectives of encapsulating microbial pigments and their application in the food industry are: Increasing shelf life, protecting the core material from undesirable environmental conditions, ease, and flexibility of handling and controlling the release time of the pigment and suppressing any type of aroma or flavor. Various methods of micro-encapsulation are available. Prominent examples used in the food industry are spray-drying, coacervation, freeze-drying and emulsion formation. There are numerous reports on encapsulated microbial pigments, such as anthocyanin, in which maltodextrin has been micro-encapsulated as the wall material, using spray-drying (177). B-Carotene has been reported to be encapsulated in modified starch as the wall material using freeze drying (178). These encapsulated colors have also been applied in food and beverage systems like yogurt, soft drinks, cake, and others, and these have shown to be stable and effective (179–181).

Nano-encapsulation or nano-emulsions are droplet size, 100 nm or less, and can also be prepared to encapsulate microbial pigments. Nano-emulsions contain three constituents, water, oil, and emulsifier. The addition of an emulsifier is the most critical step in forming a nano-emulsion, as it helps to decrease the tension between the water and oil phases of the emulsion. It also stabilizes the nano-emulsion by negating the steric hindrance and repulsive electrostatic interactions. The emulsifiers used are mostly surfactants, but proteins and lipids are also used. Compared to micro- and macro-emulsions, nano-emulsions have improved applications because of their large surface area per

unit, stronger kinetic stability and resistance to any chemical or physical change (182). Importantly, nano-emulsions, and nano-capsules are small enough to be invisible in solutions and are therefore useful vehicles for the dispersion of poorly water-soluble pigments in aqueous solutions. Creating nano-emulsions for food colorants can provide various advantages. The small sized droplets that are made in the formation of nano-emulsions provide a much greater surface area and therefore greater absorption. These nano-emulsions also are non-irritant in nature and non-toxic, making them suitable for food industry use. These can also be formulated in a wide range of formulations such as creams, liquids, sprays, and foams. Nano-emulsions create no undesired taste to the food particle and stabilize the colorant within the emulsion from all environmental conditions (183). Nano-emulsions of food colorants can significantly decrease the amount of colorant needed to obtain the desired color food particle, thereby proving to be cost effective. Various studies of nano-emulsion formation of  $\beta$ -carotene have been carried out; Yuan et al. studied the size and stability of nano-emulsions with b-carotene against temperature, pH and surfactant type. Qian et al. prepared nano-emulsions with b-carotene and stabilized them with beta-lactoglobulin, a biocompatible emulsifier (184).

## CONCLUSIONS AND FUTURE PERSPECTIVES

Natural foods are an important and growing food category that require natural ingredients and additives. Subsequently, there is a great demand to replace synthetic pigments with natural pigments in food and beverages. Microbial sources are particularly useful as they can be scaled-up and are more readily manipulated than plants or insects. Development and integration of advancements like strain development in fermentations, systems biology, metabolic, and protein engineering, can make a substantial difference in both the quality and quantity of natural food colors. Efficient fermentations include predictable yields and no external influence of the climate or environment. However, further research is required to optimize pigment characteristics, like composition and yield, by finding the most optimized parameters for growth, use of genetically modified organisms to enhance production, and also the presence of various elicitors for pigment production (185).

Metabolic engineering is useful but has its own regulatory challenges. In terms of technology, metabolic engineering can improve product yields, enable the transfer of pathways from slow growing organisms to faster growing ones, and enable directed biosynthesis of analogs of a pigment, to modify color or other properties. Cell factories can be created by utilizing CRISPER-Cas9 and heterologous expression of biosynthetic pathways from known or novel pigment producers can provide useful strategies (150, 151). Poor stability or low solubility of natural food colorants can be addressed by techniques like micro-encapsulations and nano-formulations, enabling a wider application of microbial pigments to various food matrices. Encapsulated colors are easier to handle, have better solubility and show improved stability to ambient conditions, which lead

to an increased shelf life. Nano-emulsions can be used to improve solubility and provide invisible particles that are useful in the coloring of clear and semi-clear beverages.

The current range of natural colors that can be added to foods is relatively small compared to the large range of synthetic colors. However, demand for natural foods and natural colors is increasing. The discovery of new and novel natural colors is therefore important, as is the development for technologies to improve the cost effectiveness of production and formulation of natural pigments. New natural sources to obtain pigment producing micro-organisms are required, as well as process improvements to make these strains more cost competitive with synthetic pigments. The technology required includes the development of low-cost organic substrates for the growth of pigment producing microbes, newer methods to increase the production of pigments, and stabilizing methods for improving

pigment application. Research on natural pigments should focus on obtaining a wider variety of hues, using pigments with health benefits, increasing pigment shelf life, and lowering production costs.

## AUTHOR CONTRIBUTIONS

TS drafted and edited the manuscript. CB critically revised the manuscript. SD provided critical revisions and approved the final version of the manuscript for publication.

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# Bioconversion of Beet Molasses to Alpha-Galactosidase and Ethanol

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Molasses are sub-products of the sugar industry, rich in sucrose and containing other sugars like raffinose, glucose, and fructose. Alpha-galactosidases (EC. 3.2.1.22) catalyze the hydrolysis of alpha-(1,6) bonds of galactose residues in galacto-oligosaccharides (melibiose, raffinose, and stachyose) and complex galactomannans. Alpha-galactosidases have important applications, mainly in the food industry but also in the pharmaceutical and bioenergy sectors. However, the cost of the enzyme limits the profitability of most of these applications. The use of cheap sub-products, such as molasses, as substrates for production of alpha-galactosidases, reduces the cost of the enzymes and contributes to the circular economy. Alpha-galactosidase is a specially indicated bioproduct since, at the same time, it allows to use the raffinose present in molasses. This work describes the development of a two-step system for the valuation of beet molasses, based on their use as substrate for alpha-galactosidase and bioethanol production by *Saccharomyces cerevisiae*. Since this yeast secretes high amounts of invertase, to avoid congest the secretory route and to facilitate alpha-galactosidase purification from the culture medium, a mutant in the *SUC2* gene (encoding invertase) was constructed. After a statistical optimization of culture conditions, this mutant yielded a very high rate of molasses bioconversion to alpha-galactosidase. In the second step, the *SUC2* wild type yeast strain fermented the remaining sucrose to ethanol. A procedure to recycle the yeast biomass, by using it as nitrogen source to supplement molasses, was also developed.

**Keywords:** alpha-galactosidase, invertase, *Saccharomyces cerevisiae*, beet molasses, bioconversion, ethanol

## INTRODUCTION

Molasses are viscous dark sub-products resulting from the sugar-making industry; they are rich in sucrose and contain lower amounts of other sugars like raffinose, glucose, and fructose. Due to the high sucrose content (about 50% by dry weight), at present molasses serve mostly as substrate for the industrial production of bioethanol by the yeast *Saccharomyces cerevisiae* (Akbas and Stark, 2016). The use of food industry wastes as sustainable substrates for the microbial synthesis of other biotechnological products besides ethanol, such as enzymes and other active ingredients, is increasingly gaining field in the context of the circular economy (da Silva, 2016). From this point of view, molasses provides a good carbon source for microbial cell growth (Santos et al., 2010), and the yeast biomass itself, generated in the bioprocess, may be used after a simple treatment (Ferreira et al., 2010) as source of nitrogen and B-group vitamins to complement molasses.

*Saccharomyces cerevisiae* is undoubtedly the best performing microorganism for molasses fermentation to bioethanol (Akbas et al., 2014). This yeast is also a widely employed host for heterologous protein production and secretion (Nielsen, 2013). However, there are no reports hitherto on the use of molasses as substrate with the aim of heterologous protein secretion by *S. cerevisiae*.

Alpha-galactosidases (EC. 3.2.1.22) catalyze the hydrolysis of alpha-(1,6) bonds of galactose residues in galactooligosaccharides (melibiose, raffinose, and stachyose) and complex galactomannans. Some alpha-galactosidases can also synthesize oligosaccharides by transglycosylation reactions under substrate supersaturation conditions (Spangenberg et al., 2000). Indeed alpha-galactosidases show a great variety of uses, mainly in the food industry such as improvement of separation of sucrose from beet (Linden, 1982), reduction of the content of non-digestible oligosaccharides of legume-derived food products (Katrolia et al., 2012), obtention of the low-calorie sweetener tagatose (Kim et al., 2009), improvement of rheological properties of galactomannans (Dey et al., 1993), and synthesis of prebiotics (Dai et al., 2018), but also in other sectors like pharmaceutical (Arends et al., 2017) and bioenergy (Rodrigues-Dutra et al., 2017). *S. cerevisiae* alpha-galactosidase (ScAGal), encoded by the *MEL1* gene (GeneBank X03102), is a highly glycosylated 471-amino acid extracellular protein, and the crystal structures of the complexes with melibiose and raffinose have been reported (Fernández-Leiro et al., 2010).

The cost of the enzyme actually limits the profitability of most of the above cited applications. The use of cheap sub-products, such as molasses and whey, as substrates for production of ScAGal, was previously reported and might favor the economy of the processes (Álvarez-Cao et al., 2018). The aim of this work is to develop a system for the valuation of beet molasses, based on their use as substrate for ScAGal and bioethanol production by *S. cerevisiae* in two-steps. A first step using an invertase-deficient mutant (transformed with a ScAGal over-expressing plasmid), which not only favors the secretion-purification of the enzyme due to the absence of invertase (Liljeström et al., 1991), but also increases the yield of ScAGal production, because the mutant uses the sugars present at low concentration in the molasses but not sucrose, and then the metabolism is preferentially respiratory under aerobic conditions. In the second step, the invertase wild-type strain is used to convert the sucrose to ethanol under yet established fermentation conditions.

With the engineered strains and two-step system here developed, a high rate of molasses bioconversion to alpha-galactosidase was obtained (culture conditions were statistically optimized), and sucrose was efficiently converted to ethanol. A procedure to recycle the yeast biomass, by using it as nitrogen source to supplement molasses, was also developed.

## MATERIALS AND METHODS

### Microorganisms, Expression Vectors, and Culture Media

*Saccharomyces cerevisiae* BJ3505 [*pep4::HIS3*, *prb-Δ1.6R HIS3*, *lys2-208*, *trp1-Δ101*, *ura 3-52*, *gal2*, *can1*] (Eastman Kodak

Company) was selected to construct a mutant depleted of invertase activity to be used as host for heterologous protein expression. *Escherichia coli* XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacIqZDM15 Tn10 (Tetr)]*] (Stratagene Cloning Systems) was employed for standard DNA recombinant techniques (Ausubel et al., 2003).

The plasmids YEp*MEL1* [ampr ori 2 μ *MEL1 TRP1*] and YEp*MEL1His* [ampr ori 2 μ *MEL1His TRP1*] (Fernández-Leiro, 2011), bearing the *MEL1* gene that encodes ScAGal, were chosen as expression vectors. The vector YEpFLAG-1 (Eastman Kodak Company) was used as control of the expression system.

LB [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl], and YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] were used as culture media for growth and maintenance of bacteria and yeasts, respectively. LB was supplemented with 100 mg/L ampicillin (Sigma Aldrich) for the propagation of plasmids in bacteria. A complete medium without uracil (CM-Ura) or tryptophan (CM-Trp) (Zitomer and Hall, 1976) was used for selection in *S. cerevisiae*. Two percent (w/v) bacteriological agar was added to solid media. A modified YPHSM medium and beet molasses (provided by AB Azucarera Iberia, Spain) based media were used for ScAGal production. Molasses were diluted in distilled water ratio 1:1 (v/v), to facilitate handling, and centrifuged at 10,000 rpm for 15 min to remove solid impurities. Ampicillin was filtered through 0.22 μm membrane (Sartorius AG) and the rest of media components were sterilized by autoclave at 121°C for 20 min. The composition of the media used in this work is shown in **Table 1**.

### Construction of an Invertase Defective Mutant

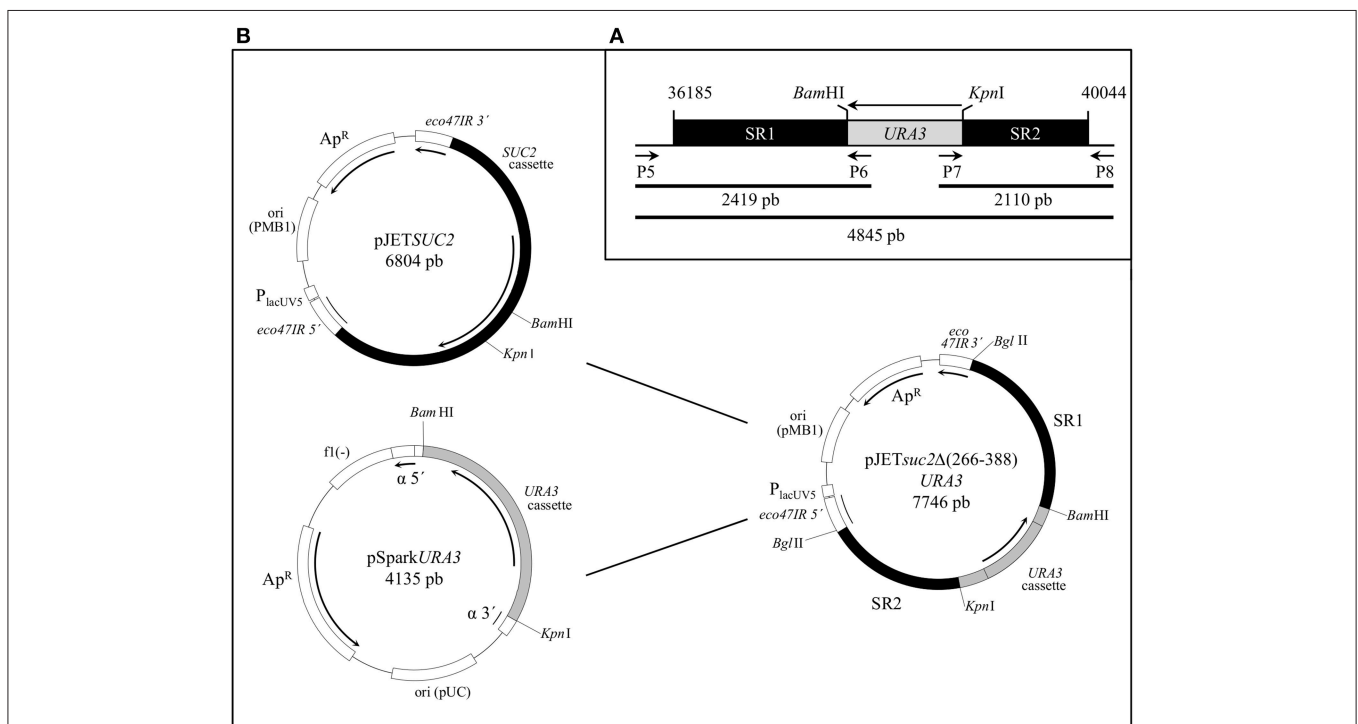
The *SUC2* gene was deleted in the *S. cerevisiae* strain BJ3505 using the integrative cassette *suc2Δ(266-388)URA3* that contains the *suc2Δ(266-388)* deletion and the *URA3* gene as selection marker (**Figure 1**). All oligonucleotides used as primers in this work are showed in **Table S1**.

### Construction of *SUC2* and *URA3* cassettes

The ORF of the *SUC2* gene was identified by means of the tool *BLAST* at *GeneBank* (<http://www.ncbi.nlm.nih.gov/>) and the homology among the different *S. cerevisiae* strains in the database. The nucleotide sequence [36185–40044] in the chromosome 9 of *S. cerevisiae* S288c (*Gene ID*: 854644; NC\_001141.2) was used and the design of the integrative cassette is showed in **Figure 1A**. A region longer than 1 Kb upstream and downstream of the *SUC2* ORF was used to compete with the marker *ura3-52* of the strain BJ3505. The fragment of 3,831 bp length corresponding to the *SUC2* cassette was amplified with the primer pairs P1–P2 (**Table S1**) from genomic DNA of the strain BJ3505 as template, by the protocol described in Hoffman (2001). A fragment of 1,308 bp flanked by the restriction sites *Bam*HI and *Kpn*I was amplified from the vector YEpIac195 (Gietz and Sugino, 1988) using the primer pairs P3–P4 (**Table S1**) to obtain the cassette *URA3* with its promoter and terminator regions. PCRs were performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific).

**TABLE 1** | Summary of the strategy of study of media and culture conditions on the production of ScAGal.

	Media	Inoculum	Strain/s
1. Expression system selection:	Modified YPHSM: 1% (w/v) yeast extract, 8% (w/v) peptone, 3% (w/v) glycerol, 2% (w/v) glucose	OD <sub>600</sub> = 0.5	BJ3505Δ <i>suc2</i> /YEp <i>MEL1</i> BJ3505Δ <i>suc2</i> /YEp <i>MEL1</i> His BJ3505/YEp <i>MEL1</i> BJ3505/YEp <i>MEL1</i> His
2. Evaluation beet-molasses media:	YR: 1% (w/v) yeast extract, 8% (v/v) beet molasses PR: 2% (w/v) peptone, 8% (v/v) beet molasses	OD <sub>600</sub> = 2	BJ3505Δ <i>suc2</i> /YEp <i>MEL1</i> BJ3505Δ <i>suc2</i> /YEpFLAG-1 BJ3505/YEp <i>MEL1</i> BJ3505Δ <i>suc2</i> /YEpFLAG-1
3. Recycling of biomass:	YR: 1% (w/v) yeast extract, 8% (v/v) beet molasses YR <sub>aut</sub> : 0.5% (v/v) autolyzed biomass, 8% (v/v) beet molasses	OD <sub>600</sub> = 4	BJ3505Δ <i>suc2</i> /YEp <i>MEL1</i>
4. Growth kinetic and conversion substrates to products:	YR: 1% (w/v) yeast extract, 8% (v/v) beet molasses	OD <sub>600</sub> = 4	BJ3505Δ <i>suc2</i> /YEp <i>MEL1</i> BJ3505/YEp <i>MEL1</i>

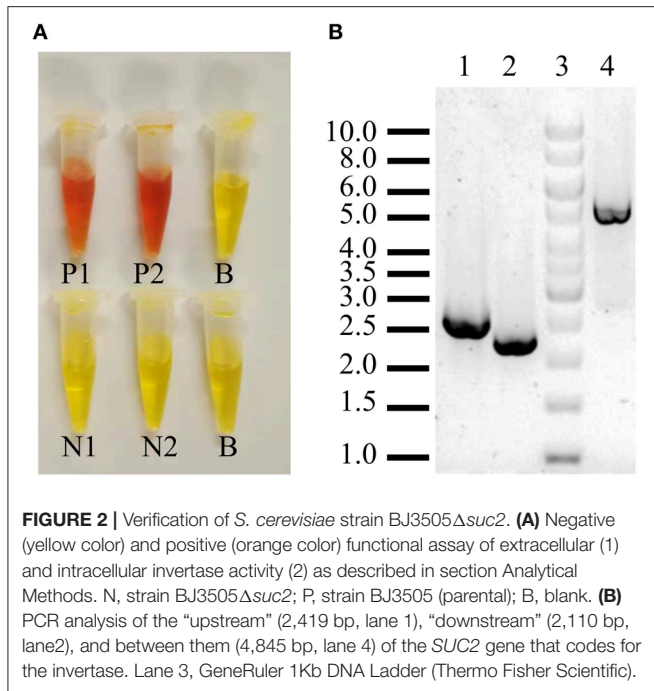


**FIGURE 1** | Construction of *S. cerevisiae* strain BJ3505Δ*suc2*. **(A)** The sequence [36,185–40,044] bp of chromosome 9 of *S. cerevisiae* (Gene ID: 854644; NC\_001141.2) containing the *SUC2* ORF was chosen to carry out the *suc2*Δ(266–388) deletion by replacement with the *URA3* cassette between sites *Bam*HI and *Kpn*I. SR1 and SR2 are the chromosomal recombination sequences and the primers pairs P5-P6, P7-P8, and P5-P8 are used to obtain the fragments 2,419, 2,110, and 4,845 bp, respectively to check the chromosomal integration. **(B)** Physical maps of the plasmids constructed to generate strain BJ3505Δ*suc2*: the *URA3* cassette from pSpark*URA3* was cloned between the *Bam*HI-*Kpn*I sites of pJETSUC2 to obtain pJETSuc2Δ(266–388)*URA3*, which was digested with *Bgl*II to release the *suc2*Δ(266–388)*URA3* cassette that was integrated through SR1 and SR2 sequences into genome of the origin strain.

### Construction of Plasmids pJETSUC2, pSparkURA3, and pJETSuc2Δ(266–388)URA3

The PCR products obtained from amplification of *SUC2* and *URA3* cassettes were cloned into vectors pJET1.2/*blunt* (CloneJET PCR Cloning Kit, Thermo Fisher Scientific) and pSpark IV (pSpark DNA Cloning System, Canvax Biotech) respectively, following the protocol recommended by each supplier, to create the plasmids pJETSUC2 and pSparkURA3.

The *Bam*HI-*Kpn*I fragment of 1,308 bp from pSparkURA3 was cloned between the *Bam*HI-*Kpn*I sites of pJETSUC2 into chemically competent XL1-Blue cells to construct the plasmid pJETSuc2Δ(266–388)URA3 with the recombination sequences SR1 (1,962 bp) and SR2 (1,503 bp) that allowed the chromosomal integration (Figure 1B). All the plasmids generated in this work were propagated and extracted from the bacterial cells using GeneJET Plasmid Miniprep Kit (Thermo



Fisher Scientific). The inserts were identified by PCR of transformant colonies using DreamTaq polymerase (Thermo Fisher Scientific) and restriction analysis, and correct sequences confirmed by sequencing (Servizos de Apoio á Investigación, Universidade da Coruña).

### Integration of the *suc2* $\Delta$ (266–388)*URA3* cassette in the BJ3505 genome

The plasmid pJET*suc2* $\Delta$ (266–388)*URA3* was digested with *Bgl*II to separate the cassette *suc2* $\Delta$ (266–388)*URA3* and further cloning in BJ3505 competent cells transformed by the lithium acetate method (Ito et al., 1983). Chromosomal integration succeeded by two homologous recombination points provided by SR1 and SR2 sequences. Recombinants were selected by growth in CM-Ura and the invertase-deficient strain, named BJ3505 $\Delta$ *Suc2*, was verified by functional and PCR analysis using DreamTaq polymerase (Thermo Fisher Scientific) (Figure 2).

### Transformation and Inoculum Preparation for ScAGal Production Cultures

The strains BJ3505 $\Delta$ *Suc2* and BJ3505 were transformed with plasmids YEpFLAG-1, YEp*MEL1*, and YEp*MEL1*His, by a modification of the lithium acetate method (Chen et al., 1992). Transformants were selected on solid CM-Trp after 48 h at 30°C. A single colony was taken to prepare a liquid CM-Trp pre-culture up to an optical density at 600 nm (OD<sub>600</sub>) of 5 (stationary phase) at 30°C and 250 rpm for 72 h, which was used to inoculate up to units OD<sub>600</sub> corresponding to each production culture tested, as described above.

### Influence of Media and Culture Conditions on ScAGal Production

Preliminary studies on cell growth determined by optical density at 600 nm (Ausubel et al., 2003) and ScAGal production from strains BJ3505 $\Delta$ *suc2* and BJ3505 were performed with PR, YR, and YR<sub>aut</sub> media (Table 1). Beet molasses were added at the desired concentration, determined as total sugars percentage, and supplemented with yeast extract or peptone to provide nitrogen source.

Yeast extract coming from biomass of ended-cultures was prepared according to a modification of the autolysis method described by Belem et al. (1997). Yeast cells were centrifuged (5,000 rpm, 15 min, 4°C), resuspended 1:2 (w/v) in 0.1M KH<sub>2</sub>PO<sub>4</sub> (pH 6.5), and autolyzed at 50°C and 150 rpm for 30 h. Supernatant was recovered by centrifugation (10,000 rpm, 15 min), cell debris was resuspended 1:10 in the same buffer and disintegrated by sonication (Sonic Vibra cell) for 10 min at 7 s intervals to facilitate the release of the remaining soluble cellular material in a second supernatant recovered by centrifugation. Both supernatants were mixed and added at 0.5% (v/v) to supplement the culture media. The RNA released from the final autolysate was analyzed by 1.5% (w/v) agarose gel electrophoresis using 1% (w/v) commercial yeast extract as control (Figure S1A).

Cultures were inoculated, from the pre-cultures of transformed strains described above, by triplicate in flasks containing 20% volume of medium, and incubated at 30°C and 250 rpm. A modified YPHSM medium (Table 1) inoculated to obtain an initial OD<sub>600</sub> of 0.5 was used to statistically compare the expression systems YEp*MEL1* and YEp*MEL1*His by analysis of variance (ANOVA) with 95% confidence intervals. Profiles of sugar consumption and products formation in the molasses-based media YR and PR were initially determined from cultures inoculated at OD<sub>600</sub> of 2. Cultures in YR and YR<sub>aut</sub> media inoculated at OD<sub>600</sub> of 4 were used to compare the assimilation of the carbon source with commercial yeast extract and autolyzed biomass, respectively. Growth kinetics and substrate to products conversion was evaluated from YR cultures inoculated at OD<sub>600</sub> of 4. In this case, when the consumption of sugar was negligible, half of each culture volume was separated while the other half was maintained in the same conditions. Under sterile conditions, the volume separated of each culture was centrifuged (5,000 rpm, 10 min, 4°C), the supernatant was refreshed with 1% (w/v) yeast extract and inoculated at the same initial OD<sub>600</sub> with cells coming from the yeast pellet. Cultures were stopped after 84 h and the last step was repeated but only with the BJ3505 $\Delta$ *suc2* cultures and using as inoculum the BJ3505 at the same initial OD<sub>600</sub>. Samples were taken at different incubation times to determine biomass, residual sugars, ethanol, and enzyme activities (invertase and alpha-galactosidase). A summary of the strategy of study of media and culture conditions is shown in Table 1.

### Microscopy

An optical microscope (Nikon Eclipse 50) was used to observe directly, without fixation, the cells from the collected culture samples. For observation under



**TABLE 2** | Experimental domain and codification of the independent variables in the CCD to the optimization of the ScAGal production by BJ3505 $\Delta$ suc2/YEpMEL1.

Real values	Coded values <sup>a</sup>				
	-2	-1	0	1	2
Beet molasses (%), X <sub>1</sub>	11	12.5	14	15.5	17
Yeast extract (%), X <sub>2</sub>	1	1.5	2	2.5	3
Inoculum size (OD <sub>600</sub> ), X <sub>3</sub>	0.5	2.5	4.5	6.5	8.5
Culture time (h), X <sub>4</sub>	36	60	84	108	132

<sup>a</sup> $x_i = (X_i - X_0) / \Delta X_i$ ,  $i = 1, 2, 3, 4$ ; where  $x_i$  and  $X_i$  are the coded and real values of the independent variable  $i$ ,  $X_0$  is the real value of the independent variable  $i$  at the central point, and  $\Delta X_i$  is the step change value.

transmission electron microscopy (TEM) (Servizos de Apoio á Investigación, Universidade da Coruña), the cells were fixed in 100 mM sodium cacodylate buffer pH 7.2 as described in Bauer et al. (2001).

## Optimization of ScAGal Production by the Surface Response Methodology

Statistical optimization of beet molasses based cultures for ScAGal production was performed using the response surface methodology (RSM) with the strain BJ3505 $\Delta$ suc2/YEpMEL1. The experimental factors selected as independent variables were 4: concentration of molasses and yeast extract, inoculum size, and culture time. The dependent variable or response was extracellular alpha-galactosidase activity. A central composite design (CCD) was applied to study the effects and interactions among the four factors at five different levels. Their coded values were  $-\alpha$ ,  $-1$ ,  $0$ ,  $+1$ ,  $+\alpha$ , being  $\alpha = 2^{k/4}$ ,  $k$  the number of independent variables and  $0$  the central point. **Table 2** shows the levels and real values of the factors, calculated according to the equation described by de Faria et al. (2013). The RS was adjusted to a polynomial second order equation that correlates the measured response with the independent variables (Dilipkumar et al., 2014), and the optimum value of the CCD was obtained solving the regression equation and analyzing the surface response contour plots. An ANOVA with 95% confidence intervals was performed to determine the significance of the effects.

## Bioreactor Operation

Bioreactor cultures were run in a Biostat-MD (Braun-Biotech) 2 L fermentor, 1 L working volume, with pH control to evaluate its effect on ScAGal production. For this, the fermentations were carried out without and with adjustment to pH 6 during the course of the culture. Medium composition and inoculum size were selected from the results of the CCD described above. Bioreactor was sterilized at 121°C for 30 min, and then aseptically inoculated and supplemented with 300 mg/L ampicillin. Culture conditions were 30°C, 2 L/min air flow and 300 rpm, for 120 h (Ausubel et al., 2003). Sterilized 1 M NaOH or 1 M HCl were added as needed to adjust pH. Samples were taken at regular time intervals to determine yeast biomass, extracellular alpha-galactosidase activity, and conversion of substrates into

secondary metabolites. Plasmid stability was determined as percentage of viable Trp<sup>+</sup> colonies, after seeding diluted samples of the culture on Petri dishes with CM and CM-Trp and counting colony forming units by the following equation,

$$\text{Percentage plasmid stability (\%)} = \frac{\text{Colony Forming Units \{CM - Trp\}}}{\text{Colony Forming Units \{CM\}}} \times 100$$

## Analytical Methods

### Molasses Sugar and Protein Content

The sugars present in the diluted molasses used to prepare the culture media were identified and quantified by High-Performance Liquid Chromatography (HPLC) using an external standard formed by known concentrations of raffinose, sucrose, glucose, and fructose ranging 4–0.06 mg/mL. An estimate of the nitrogen fraction in the diluted molasses according to the protein content was measured by the Bradford's method using DC Protein Assay Kit (Bio Rad) and bovine serum albumin as standard.

### Biomass, Residual Sugars, and Ethanol Determination

Cell growth in shaken flasks was measured as units of OD<sub>600</sub> with an UV-Visible spectrophotometer (Biospectrometer Kinetic Eppendorf) to express the biomass generated. Cell-free medium was used to measure ethanol, with the enzymatic assay Ethanol UV method (NZYTech), and residual sugar. The latter was quantified indirectly using the DNS method (Miller, 1959) by means of the analysis of reducing sugars content in the samples before and after hydrolysis at 95°C for 5 min with 18.5% (w/v) HCl followed by neutralization with 25% (w/v) NaOH. For cultures in bioreactor, biomass was quantified as dry weight from 5 mL samples, after being centrifuged at 5,000 rpm for 5 min, washed with distilled water, centrifuged again, and dried at 105°C until constant weight. The supernatant was used to measure sugar consumption, ethanol, and other metabolites formation by HPLC using an external standard composed by known concentrations of raffinose, sucrose, galactose, glucose, fructose, glycerol, and ethanol ranging 4–0.06 mg/mL.

### HPLC Analysis

Sugar Pack Waters (6.5 × 300 mm) column and Refractive Index Detector (Cienytech) were used. Samples were previously clarified with cartridges HyperSep Silica SPE Column (Fisher Scientific) to avoid interferences due to pigments and other impurities coming from molasses. Column temperature 80°C, detector temperature 37°C, and sensitivity 32 were the running conditions using as mobile phase 100 μM EDTA-Calcium (Sigma Aldrich) at flow rate of 0.5 mL/min. Eluted compounds were identified and quantified using sorbitol (1 mg/mL) as internal standard, since it does not interfere with the retention times of the compounds under study (Xu et al., 2015).

### Enzyme Activity Assay

Extracellular alpha-galactosidase activity was measured by the method of Ryan et al. (1998), incubating the samples with

a 10 mM solution of the synthetic substrate *p*-nitrophenyl- $\alpha$ -galactose (Sigma Aldrich) in McIlvaine buffer (pH 4) at 40°C. At two consecutive time intervals, the reaction was stopped with 0.5 M Na<sub>2</sub>CO<sub>3</sub> and released *p*-nitrophenol was measured at 400 nm (molar extinction coefficient: 18.20 mmol<sup>-1</sup>.cm<sup>-1</sup>). Invertase activity, extracellular, and intracellular was measured from supernatant and sediment of culture samples, respectively, after permeabilization with 15% (v/v) chloroform and 0.01% (w/v) SDS. The samples were incubated with 100 mM sucrose in 50 mM acetate buffer (pH 5) for 10 min at 40°C, and reducing sugars were determined by the DNS method (Miller, 1959). One enzyme unit (U) is defined as the amount of enzyme that releases one  $\mu$ mol of product per minute under assay conditions.

### Statistical Analysis

Statistical data treatment was done using the StatGraphics Centurion XVI package and graphics construction was performed with the program SigmaPlot 12.0. Significance of data was evaluated with Student's *t*-test and results were considered significant for  $p \leq 0.05$ .

## RESULTS

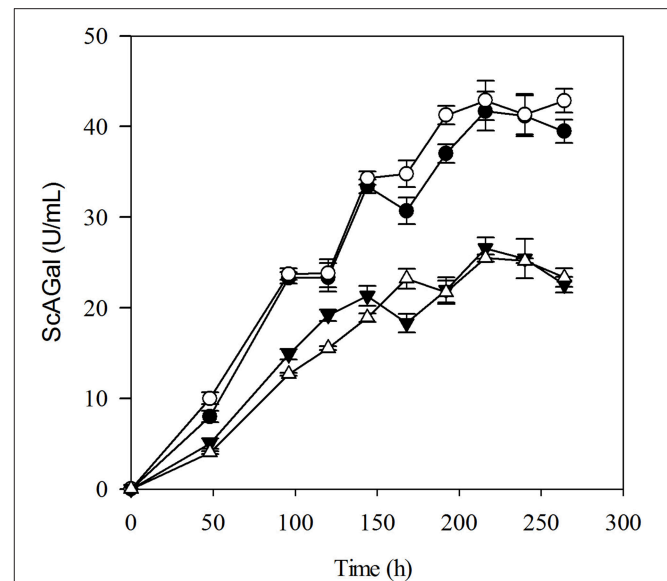
### Construction of an Invertase Defective Mutant

The strain BJ3505 $\Delta$ *suc2* was constructed by the procedure described in the Materials and Methods section. The correctness of the mutation was verified by functional measurement of invertase activity that was fully depleted in the mutant (Figure 2A) and by PCR analysis (Figure 2B, see also Figure 1A) that showed bands of the expected size in function of the chromosomal integration.

### Approach to a Medium for ScAGal Production Based on Beet Molasses

Cultures in YPHSM of the strains BJ3505 $\Delta$ *suc2* and corresponding wild type BJ3505, transformed with the plasmids YEpMEL1His or YEpMEL1, with and without affinity purification tag, respectively, were performed to select the best ScAGal expression system. YPHSM is the recommended medium for heterologous protein expression with the YEpFLAG-1 system (Eastman Kodak Company) that was used to construct the plasmids (Fernández-Leiro, 2011). Growth was similar in the four cases (OD<sub>600</sub> = 90 ± 10; data not shown), and maximum extracellular alpha-galactosidase activity was similar for the two strains but dependent on the plasmids, 43 and 25 U/mL for YEpMEL1 and YEpMEL1His, respectively, after 216 h of incubation (Figure 3). Although the statistical analysis shows that there is not a direct correlation between ScAGal secretion and lack of invertase in glucose (YPHSM) medium (Table S2), the advantage of obtaining alpha-galactosidase preparations not contaminated with invertase is clear. Therefore, BJ3505 $\Delta$ *suc2*/YEpMEL1 was selected as an improved ScAGal expression system for the following experiments.

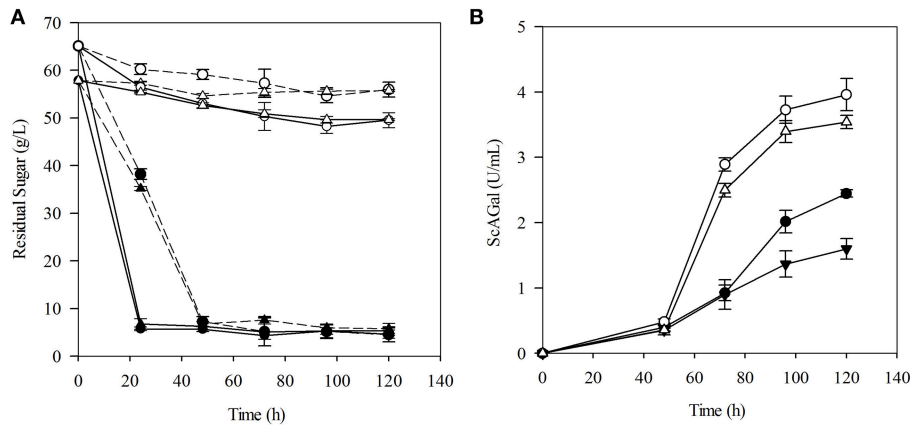
Raw molasses used in this work contained 59.7% sucrose, 2.9% raffinose, 2.4% fructose, 1.2% glucose, and 0.3% nitrogen fraction.



**FIGURE 3** | Time course of extracellular alpha-galactosidase activity using a conventional culture medium. Cultures in the YPHSM medium were inoculated to an OD<sub>600</sub> of 0.5 from pre-cultures of strains BJ3505 $\Delta$ *suc2* (empty symbol) and BJ3505 (full symbol) transformed with the plasmids YEpMEL1 (circle), and YEpMEL1His (triangle).

Beet molasses are limited in biotin (vitamin B7) and other sources of nitrogen, and yeast biomass can be valuable for using as nitrogen and vitamins supplement for microbial growth (Ferreira et al., 2010). Then, the effects on sugar consumption and ScAGal extracellular production of molasses supplementation with yeast extract and peptone using the YR and PR media, respectively, were assayed and are shown in Figure 4. Results prove that, as expected due to the  $\Delta$ *suc2* mutation, the strain BJ3505 $\Delta$ *suc2* uses only 10–20% of the sugars provided by the media, while the strain BJ3505 (with intact *SUC2* gene) uses the 90% at the same culture time. An increase of sugar consumption rate was observed for strains transformed with YEpMEL1 due to alpha-galactosidase activity (Figure 4A). From Figure 4B, it is inferred that in YR and PR media, BJ3505 $\Delta$ *suc2*/YEpMEL1 produces more extracellular ScAGal than BJ3505/YEpMEL1 does, i.e., indeed invertase depletion favors ScAGal secretion in sucrose-rich medium, and that yeast extract can be used as supplement instead of peptone, since ScAGal production by BJ3505 $\Delta$ *suc2*/YEpMEL1 is quite similar with both supplements. Furthermore, the recycling of yeast biomass as molasses supplement was analyzed and it was found that the autolysis treatment provides a source rich in low molecular weight nucleotides, as is well-documented (Belem et al., 1997). Although the results also confirmed very close values of sugar consumption and extracellular ScAGal production (Figure S1B), we continued to use commercial yeast extract for the following experiments, as it is more standard and reproducible among laboratories.

Figure 5 shows culture profiles of BJ3505 $\Delta$ *suc2*/YEpMEL1 and the corresponding wild type BJ3505/YEpMEL1 in 8% beet molasses supplemented with 1% yeast extract. It is



**FIGURE 4 |** Evaluation of culture media based on beet molasses for ScAGal production. Time course of residual sugar **(A)** and extracellular alpha-galactosidase activity **(B)** by BJ3505 $\Delta$ suc2 (empty symbol) and BJ3505 (full symbol) in culture media YR (triangle) and PR (circle). The solid and dashed lines correspond to the strains transformed with the plasmid YEpMEL1 and YEpFLAG-1, respectively. The media YR and PR were prepared with 8% beet molasses supplemented with 1% yeast extract or 2% peptone and the cultures were inoculated to  $OD_{600} = 2$ .

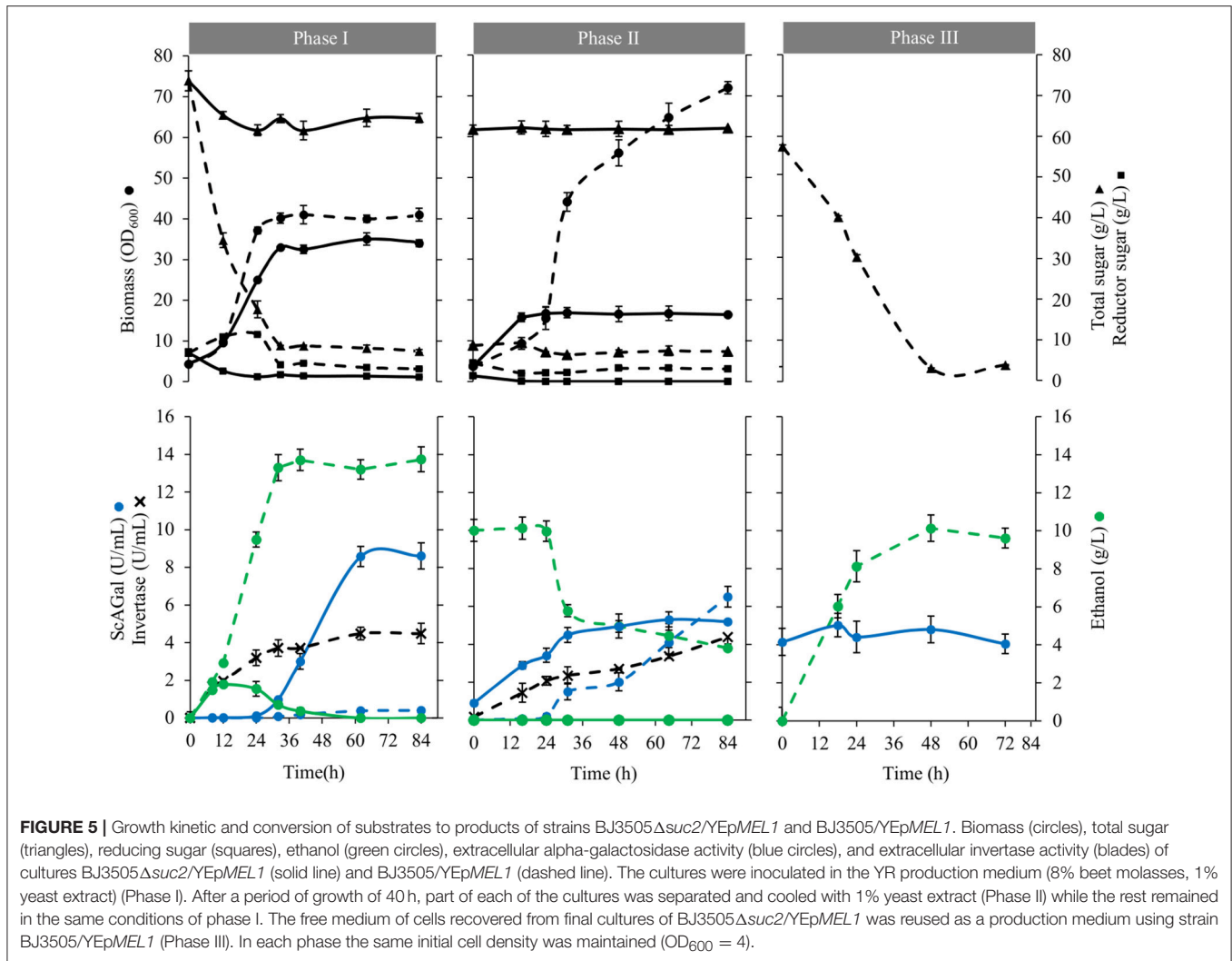
observed that both strains reach similar growth levels but while BJ3505/YEpMEL1 consumed 88% sugars (native invertase hydrolyzed sucrose) and produced 13 g/L of ethanol, BJ3505 $\Delta$ suc2/YEpMEL1 consumed 12% sugars producing only 1.8 g/L ethanol that was later metabolized as carbon source (Phase I). After 40 h of incubation, all available sugar was consumed by both strains and a part of the cultures was supplemented again with 1% yeast extract, which caused that BJ3505/YEpMEL1 shifted to use ethanol as carbon source and to secrete ScAGal, reaching a biomass four-fold higher than BJ3505 $\Delta$ suc2/YEpMEL1 that continued secreting ScAGal (Phase II). Finally, the cell free medium at the end of the BJ3505 $\Delta$ suc2/YEpMEL1 culture was inoculated with BJ3505/YEpMEL1 that converted the sucrose into ethanol (Phase III). So, the metabolism of BJ3505/YEpMEL1 is fermentative and produces ethanol from sucrose in 8% molasses, even under aerated conditions, known as Crabtree effect (Marques et al., 2015). However, BJ3505 $\Delta$ suc2/YEpMEL1 shows a respiratory metabolism, probably due to the lower amount of sugars available since this strain cannot use sucrose, and metabolizes the rest of sugars present in molasses producing alpha-galactosidase more efficiently.

Moreover, both strains BJ3505 $\Delta$ suc2/YEpMEL1 and BJ3505/YEpMEL1 show morphological differences depending on the culture phase (Figure 6). By optical microscopy, the presence of particles around the vacuole is observed in BJ3505/YEpMEL1, while no vacuole is visible in BJ3505 $\Delta$ suc2/YEpMEL1 and there are fewer particles in the cytoplasm (Phase I, Figure 6A). However, when BJ3505/YEpMEL1 starts to use ethanol as carbon source, the morphology of both strains is similar (Phase II, Figure 6A). The TEM images show in more detail the intense cytoplasmic activity in BJ3505 $\Delta$ suc2/YEpMEL1 possibly as a result of the production of heterologous protein compared to the strain of origin (Phase I, Figure 6B).

## Optimization of ScAGal Production by BJ3505 $\Delta$ suc2/YEpMEL1 From Beet Molasses

The matrix of CCD shown in Table 3 defined a set of 30 experiments, including six replicas of the central point to estimate the experimental error of the production technique. The corresponding results are also shown. Following, the surface response methodology (SRM) was applied to the experimental observed data in order to adjust the response function (extracellular alpha-galactosidase activity) and reveal the simultaneous influence of the four studied variables (molasses concentration, yeast extract concentration, inoculum size, and incubation time) on it. The best condition for ScAGal production corresponded to experiment number 13 that reached an extracellular alpha-galactosidase activity of 17 U/mL. In contrast, the worst condition (experiment 23) showed an activity value as low as 0.001 U/mL.

To validate the significance of the model obtained, the ANOVA presented in Table 4 was performed. The Pareto graphic analysis of the model shows the significance of the influence of the variables on the response (Figure 7A). The effects statistically not significant at the 95% confidence level were excluded from the model (and do not appear in Table 4), except yeast extract concentration that was the closest to the significance limit. Inoculum size and incubation time showed a positive effect on the response, whereas molasses concentration effect was negative. The effect of yeast extract concentration was smaller than the other three variables as corresponds to its lower significance level. Moreover, the representation of the single interaction that was significant, between inoculum size and incubation time (Figure 7B), shows that the increase of the inoculum size provokes a higher increase of the response if the incubation time also increases. The correlation coefficient ( $R^2$ ) that explains the 86.8% of the variation of the response and the  $p$ -value >



0.05 for the lack of fit test prove that the regression model is significant and can be used to describe the production of ScaGal into the defined experimental domain. Therefore, the following equation describes the relationship between response and variables: Extracellular alpha-galactosidase activity ( $Y$ ) =  $3.78 - 1.70x_1 + 0.58x_2 + 2.72x_3 + 3.98x_4 + 1.23x_1 x_1 + 0.85x_3 x_3 + 1.67C_3x_4$ .

SRM and contour plot analysis reinforce that the effect of increasing incubation time ( $T$ ) is more positive as inoculum size ( $I$ ) increases (Figure 8A), and also when molasses concentration ( $M$ ) decreases (Figure 8B). The combination of maximum  $I$  and minimum  $M$  also drives to an increase in ScaGal production (Figure 8C), while the effect of yeast extract ( $YE$ ) is scarcely relevant in the experimental domain studied (Figures 8D–F).

The maximum ScaGal production value is obtained in the experimental domain conditions  $x_1 = -2$ ,  $x_2 = 0$ ,  $x_3 = +2$ ,  $x_4 = +2$ , which correspond to the real values 11%  $M$ , 2%  $YE$ , 8.5OD<sub>600</sub>  $I$ , and 132 h  $T$ , respectively. According to the estimated model, an increase in  $YE$  ( $x_2 = 0$ ,  $X_2 = 2\%$ ) would slightly increase the ScaGal production (above 4%) compared to the value  $x_2$

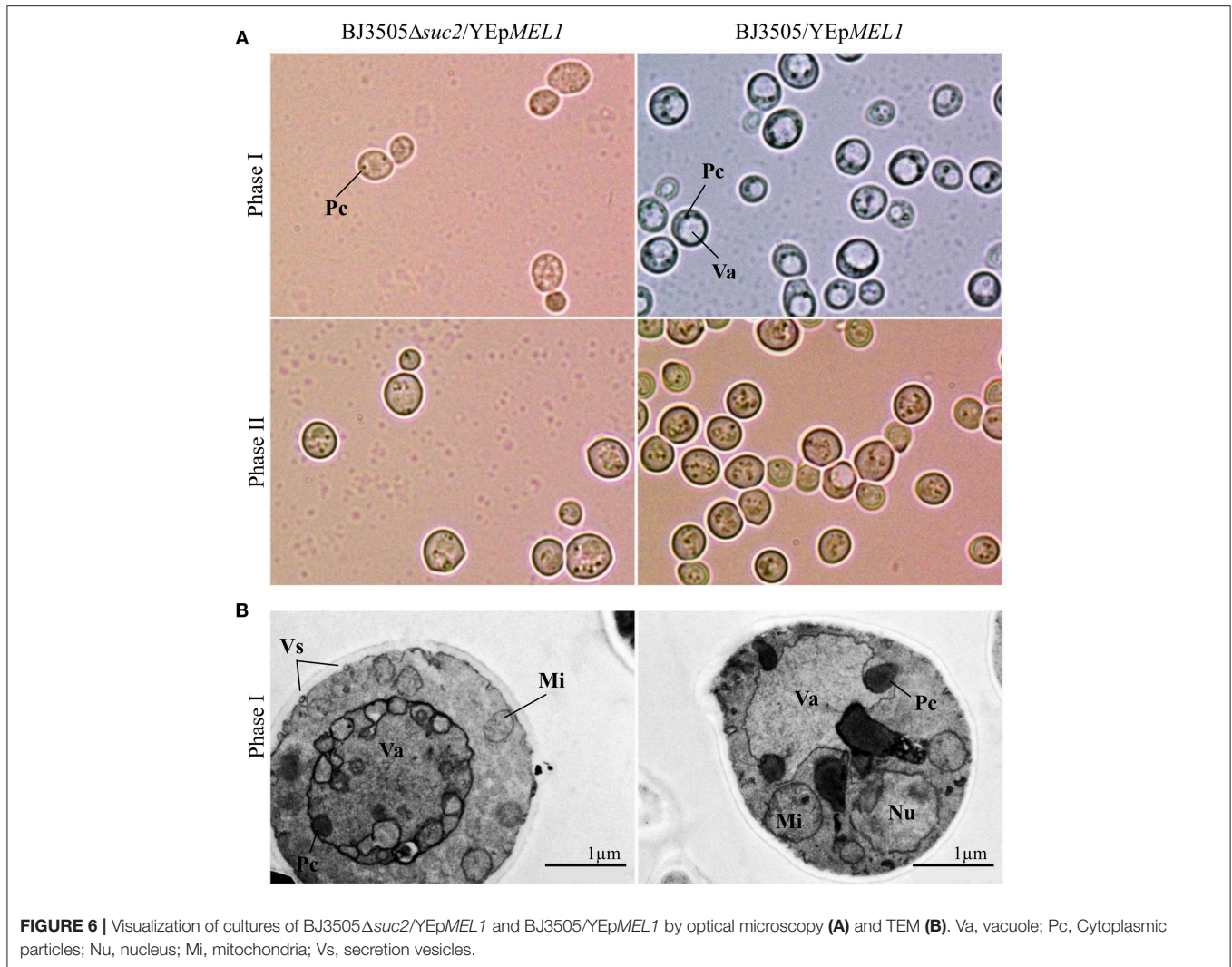
= -2 ( $X_2 = 1\%$ ) of the same variable, keeping the rest of the conditions optimized. Therefore, we decided to use 1%  $YE$  to economize the productive process and since it is also the concentration usually used in commercial culture media. Under these conditions, the strain BJ3505Δ*suc2*/YEpMEL1 yielded the maximum extracellular alpha-galactosidase activity of 24 U/mL (Figure S2). This is a value close to predicted by the model (34 U/mL) and represents an improvement of the 72% with respect to the ScaGal production observed in the first studies performed in this work.

Therefore, in addition to being sustainable and low-cost, we have proved that molasses are also promising substrates for ScaGal production with the BJ3505Δ*suc2*/YEpMEL1 strain engineered in this work.

### Scale-Up to 2-L Bioreactor

The strain BJ3505Δ*suc2*/YEpMEL1 was cultured in 2-L bioreactors with 1-L working volume of optimized YR (beet molasses-yeast extract) medium under controlled pH conditions. *S. cerevisiae* strains grow well between pH 4.5





**FIGURE 6** | Visualization of cultures of BJ3505Δsuc2/YEpMEL1 and BJ3505/YEpMEL1 by optical microscopy (A) and TEM (B). Va, vacuole; Pc, Cytoplasmic particles; Nu, nucleus; Mi, mitochondria; Vs, secretion vesicles.

and 6.5 (Walker and Stewart, 2016), and we decided to maintain bioreactors at pH 6 since it slightly improved the ScAGal production according to our previously observed experiments (Álvarez-Cao, 2017). **Figure 9A** shows the monitoring of bioreactors using optimized YR without (Bioreactor 1) and with (Bioreactor 2) adjustments at pH 6 during the culture time, where we found unexpected results that had not been considered before. First, cellular growth stopped at 72 h in both cases, but biomass and extracellular alpha-galactosidase activity were approximately double when the culture was maintain at pH 6, reaching about 20 g/L and 30 U/L, respectively, at 120 h of culture. Second, the pH value suddenly increased to pH 9 when it was not adjusted during the course of the culture. Apart from ScAGal, other metabolites were produced depending on the culture phase, shown in **Table 5**. Thus, during the first 24 h, the strain used all the glucose and only 73% (Bioreactor 1) or 82% (Bioreactor 1) of the fructose to produce ethanol and glycerol as co-metabolites. At the same time, galactose appears in the medium coming from 5% (Bioreactor 1) to 25% (Bioreactor 2) of the hydrolysis of raffinose by the action of ScAGal. Galactose concentration was

maintained constant thereafter in both cultures, meaning that the strain prefers to use other monosaccharides (Marques et al., 2015). On the other hand, in the bioreactor without adjustment at pH 6 (Bioreactor 1; **Figure 9A**), from 24 to 48 h the strain metabolism was preferentially fermentative producing 8 g/L ethanol and 1.6 g/L glycerol while ScAGal activity was kept constant. During the following 48–96 h, strain's metabolism shifted to respiratory using ethanol and glycerol as carbon sources, and ScAGal expression sharply increased. The next 24 h, the production of ethanol and glycerol re-started and ScAGal activity reached 20 U/mL at 120 h of culture. However, when the bioreactor pH was maintained at 6 (Bioreactor 2; **Figure 9A**), the strain metabolism was respiro-fermentative during all culture, producing ethanol, glycerol, and ScAGal until 48 h, from 48 to 72 h consumed all the previously produced ethanol and a part of the glycerol while ScAGal increased up to 20 U/mL, and from 72 to 120 h, ethanol was newly produced and levels of ScAGal reached the highest values obtained (30 U/mL). If both cultures were extended, ethanol consumption and ScAGal production could be increased. These results are superior to

**TABLE 3** | Experimental matrix according to the CCD and results observed and predicted by the MSR to the optimization of the ScAGal production by BJ3505 $\Delta$ suc2/YEpMEL1.

Exp no.	Coded values				Real values <sup>a</sup>				(U/mL) <sup>b</sup>	
	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	X <sub>4</sub>	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	X <sub>4</sub>	Observed	Estimated (Y)
1	-1	-1	-1	-1	12.5	1.5	2.5	60	0.465	1.952
2	1	-1	-1	-1	15.5	1.5	2.5	60	0.316	-1.447
3	-1	1	-1	-1	12.5	2.5	2.5	60	0.706	3.107
4	1	1	-1	-1	15.5	2.5	2.5	60	0.268	-0.292
5	-1	-1	1	-1	12.5	1.5	6.5	60	1.721	4.061
6	1	-1	1	-1	15.5	1.5	6.5	60	1.306	0.662
7	-1	1	1	-1	12.5	2.5	6.5	60	1.935	5.216
8	1	1	1	-1	15.5	2.5	6.5	60	1.451	1.817
9	-1	-1	-1	1	12.5	1.5	2.5	108	6.763	6.580
10	1	-1	-1	1	15.5	1.5	2.5	108	6.038	3.181
11	-1	1	-1	1	12.5	2.5	2.5	108	7.107	7.735
12	1	1	-1	1	15.5	2.5	2.5	108	5.440	4.336
13	-1	-1	1	1	12.5	1.5	6.5	108	17.431	15.360
14	1	-1	1	1	15.5	1.5	6.5	108	11.071	11.961
15	-1	1	1	1	12.5	2.5	6.5	108	15.709	16.515
16	1	1	1	1	15.5	2.5	6.5	108	12.478	13.116
17	-2	0	0	0	11	2	4.5	84	16.005	12.117
18	2	0	0	0	17	2	4.5	84	2.345	5.319
19	0	-2	0	0	14	1	4.5	84	1.081	2.630
20	0	2	0	0	14	3	4.5	84	8.019	4.940
21	0	0	-2	0	14	2	0.5	84	0.299	1.732
22	0	0	2	0	14	2	8.5	84	14.968	12.621
23	0	0	0	-2	14	2	4.5	36	0.001	-4.178
24	0	0	0	2	14	2	4.5	132	10.846	11.749
25	0	0	0	0	14	2	4.5	84	2.479	3.785
26	0	0	0	0	14	2	4.5	84	2.116	3.785
27	0	0	0	0	14	2	4.5	84	2.479	3.785
28	0	0	0	0	14	2	4.5	84	2.766	3.785
29	0	0	0	0	14	2	4.5	84	2.240	3.785
30	0	0	0	0	14	2	4.5	84	2.521	3.785

<sup>a</sup>X<sub>1</sub>, beet molasses (%); X<sub>2</sub>, yeast extract (%); X<sub>3</sub>, inoculum size (OD<sub>600</sub>); X<sub>4</sub>, culture time (h).

<sup>b</sup>Extracellular ScAGal activity ( $\mu$ mol.min.mL<sup>-1</sup>) to pH 4 and 40°C.

those obtained in a similar characteristics bioreactor using the rich and much more expensive medium YPHSM with 5% (w/v) glucose (a level of assimilable sugars comparable to YR medium) that yielded more biomass (25 g/L) but less ScAGal (25 U/mL) (Bioreactor 3; **Figure 9A**). So, scale-up to 1-L culture in bioreactor with pH control allowed to increase a 21% the levels of ScAGal production compared with the best results obtained using shaken flasks (**Figure S2**).

Strikingly, when cells coming from ended-cultures of both bioreactors, at pH 9 and pH 6, were seeded in solid CM-Trp medium, only those coming from pH 9 acquired pink color (Bioreactor 1; **Figure 9B**) while the second ones maintained the normal phenotype (Bioreactor 2; **Figure 9B**). This characteristic was maintained during successive re-seedings for 10 months (Re-seeding; **Figure 9B**). Moreover, pink colonies grew faster than white ones and with a peculiar morphology. On the other hand, plasmid stability resulted to be about

98% in both bioreactors, independently of pH value, after 120 h of culture.

## DISCUSSION

Molasses are highly abundant sub-products of the food industry that are hitherto being fermented to ethanol at industrial level due to their richness in sucrose. The yeasts employed are adapted strains of *S. cerevisiae*, that produces high amounts of extracellular invertase, a native enzyme that hydrolyses sucrose into glucose and fructose, and is a Crabtree positive yeast, which means that preferentially ferments high concentrations of sugars to ethanol even under aerated conditions (Marques et al., 2015).

In this work, we started from the hypothesis that a mutant strain not-producing invertase ( $\Delta$ suc2), i.e.,

unable to use sucrose, would metabolize the sugars present in molasses at lower concentration (raffinose, glucose, fructose) that could be used to produce a recombinant protein. In our case, the selected recombinant protein was ScAGal, an enzyme widely used in several industries and with large market, that would also simultaneously allow to use the raffinose present in molasses since ScAGal shows a high affinity for this sugar (Fernández-Leiro et al., 2010). The remaining sucrose could be fermented by wild type *S. cerevisiae* in a coupled second-step classical industrial process.

Figure 10 shows the scheme of a proposed productive process of ScAGal and bioethanol in the conditions optimized in this work, using beet molasses as substrate and the constructed

engineered yeast strain. In the first step, ScAGal would be obtained from cultures of the strain BJ3505 $\Delta$ suc2/YEpMEL1 in molasses medium. In the second step, bioethanol would be obtained from cultures of any wild type yeast strain, in our case the strain of origin BJ3505, in post-incubate cell-free sucrose-rich medium resulting from the first step after ScAGal recovery by tangential flow filtration (TFF). The ethanol produced in step 2 could be extracted for biofuel use, and also recycled as carbon source for ScAGal production in the step 1. Moreover, one part of the yeast biomass obtained (of each of the two strains) would serve as inoculum of the following culture, and the rest would be autolyzed and then used to supplement molasses instead of the more expensive commercial yeast extract.

During the research conducted to develop the first step of this proposed coupled process, we found several interesting observations. On the one hand, the  $\Delta$ suc2 strain produced more extracellular ScAGal from molasses than the corresponding wild type strain. We attributed this result to the relief of the secretory route due to the absence of invertase (Liljeström et al., 1991) and to the shift to a respiratory metabolism due to the inability to use sucrose (Finn et al., 2006). In addition, ScAGal became easier to purify from the culture medium without contaminating invertase. On the other hand, the  $\Delta$ suc2 strain presented some curious morphological changes. In the first place, the absence of vacuolar particles that are observed in the wild type strain growing in molasses, and that in the case of the mutant are cytosolic. Under stress conditions, the vacuole is prone to flow between non-fragmented and fragmented forms due in part to its association with other cellular organelles (endoplasmic reticulum, Golgi complex, secretory, and endocytic vesicles) as components of interconnected branched systems (Gibson et al., 2007). This change of morphology could be attributed to a stress response due to environmental factors, such as glucose depletion, tolerance to ethanol (Gibson et al., 2007; Kato et al., 2011; Grousl et al., 2013), or to the switch from fermentative to respiratory metabolism since the wild type strain growing

**TABLE 4** | ANOVA for the response surface quadratic model to the optimization of the ScAGal production by BJ3505 $\Delta$ suc2/YEpMEL1<sup>a</sup>.

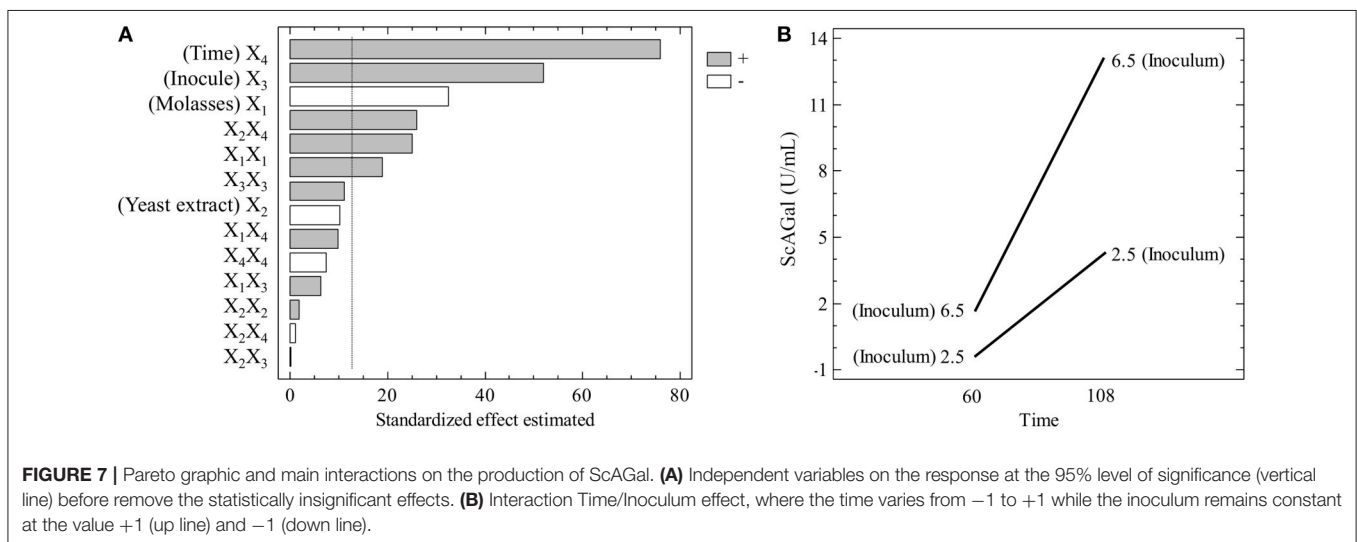
Effects <sup>b</sup>	Sum of squares	Df <sup>c</sup>	Mean square	F-ratio	p-value <sup>d</sup>
x <sub>1</sub>	69.323	1	69.323	1052.18	0.0196
x <sub>2</sub>	8.003	1	8.003	121.47	0.0576
x <sub>3</sub>	177.872	1	177.872	2699.75	0.0123
x <sub>4</sub>	380.480	1	380.480	5774.96	0.0084
x <sub>1</sub> x <sub>1</sub>	37.071	1	37.071	562.66	0.0268
x <sub>3</sub> x <sub>3</sub>	17.520	1	17.520	265.92	0.0390
x <sub>3</sub> x <sub>4</sub>	44.499	1	44.499	675.41	0.0245
Lack of Fit	110.244	17	6.485	98.43	0.0780
Pure Error	0.065	1	0.065		
Total	835.492	25			

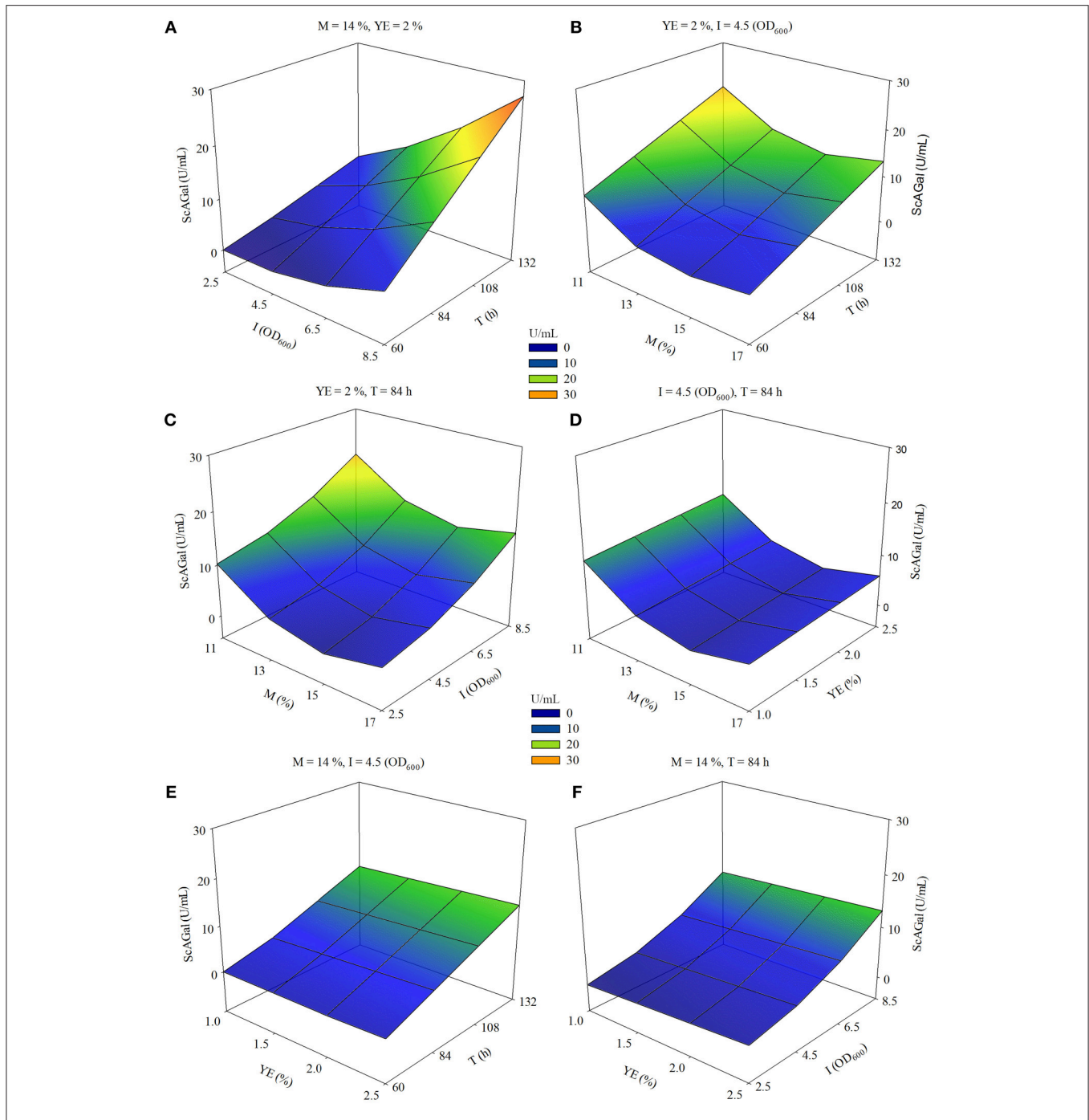
<sup>a</sup>R<sup>2</sup> = 86.80%; adjusted R<sup>2</sup> = 81.66%; standard error = 0.256; mean absolute error = 1.74.

<sup>b</sup>Linear effects (x<sub>1</sub>, molasses; x<sub>2</sub>, yeast extract; x<sub>3</sub>, inoculum size; x<sub>4</sub>, culture time), quadratic effects (x<sub>1</sub>x<sub>1</sub>; x<sub>3</sub>x<sub>3</sub>), and interaction effect (x<sub>3</sub>x<sub>4</sub>).

<sup>c</sup>Df, Degrees of freedom.

<sup>d</sup>p ≤ 0.05 denotes a statistically significant difference.





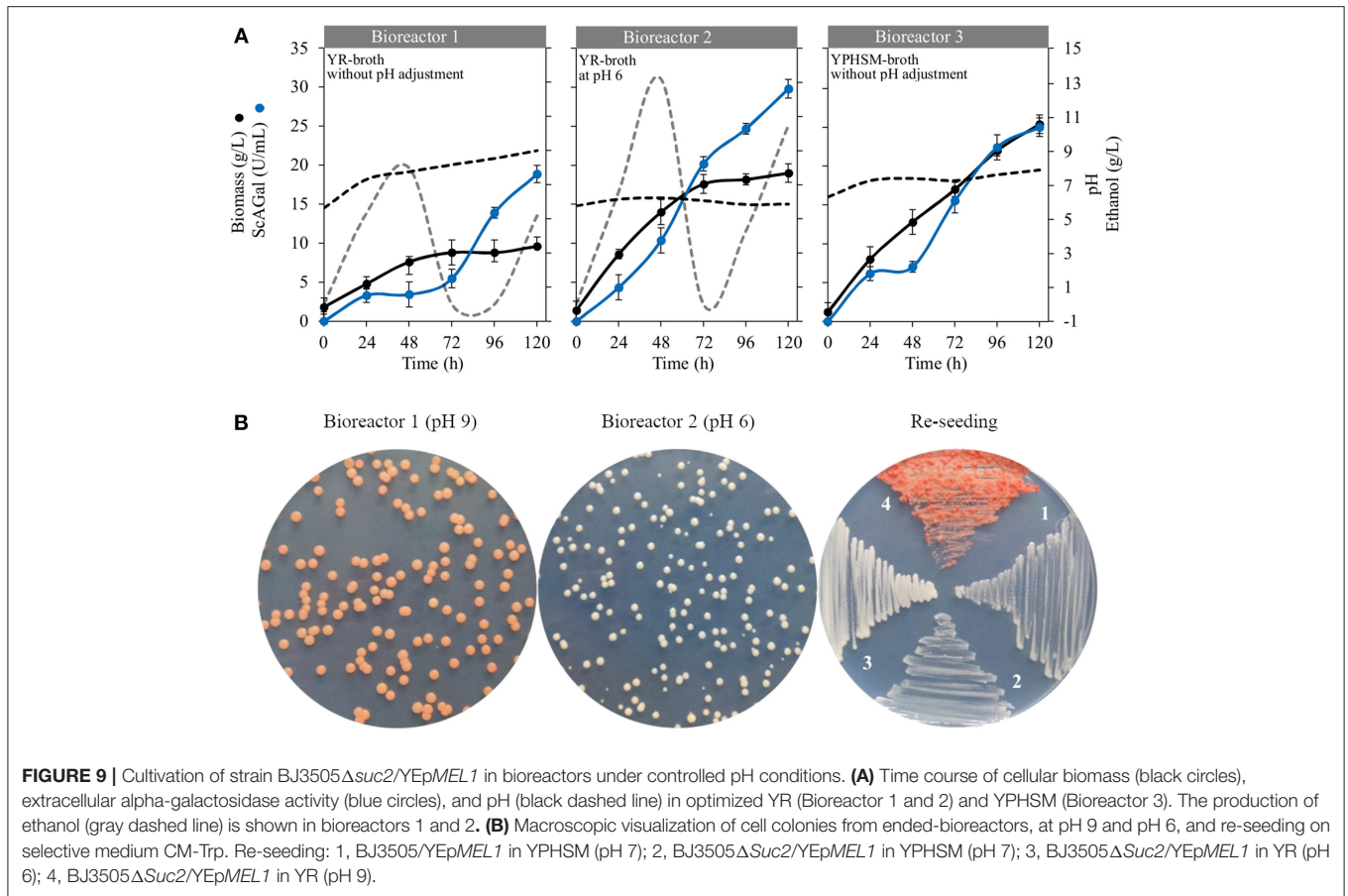
**FIGURE 8 |** Response surface-contour plots for extracellular ScAGal production by the strain BJ3505 $\Delta$ suc2/YEpMEL1 on beet molasses based medium using RSM. Estimated extracellular alpha-galactosidase activity (ScAGal) as function of (A) inoculum size (I) and time (T); (B) beet molasses (M) and time (T); (C) beet molasses (M) and inoculum size (I); (D) beet molasses (M) and yeast extract (YE); (E) yeast extract (YE) and time (T); (F) yeast extract (YE) and inoculum size (I). The values of the third and fourth variables were remained constant at level 0.

in ethanol does not present such vacuolar particles. In the second place, the  $\Delta$ suc2 strain gains a pink color in a pH-dependent manner that could be related to the synthesis of carotenoid pigments by yeast as has also been reported in the literature for other microorganisms (Querijero-Palacpac et al., 1990; Schmidt et al., 2011). However, the molecular mechanism

of these morphological changes merits to be the subject of further research.

It is well-known that high concentrations of sucrose exert hyperosmotic stress on *S. cerevisiae* which synthesizes antioxidant and osmoregulatory molecules to avoid lethal injuries, such as trehalose and glycerol, among others (Gibson





**TABLE 5 |** HPLC analysis to quantify the conversion of substrates to products during the cultivation of bioreactors with strain BJ3505 $\Delta$ suc2/YEpMEL1<sup>a</sup>.

g/L	YR	Bioreactor 1 <sup>b</sup>					Bioreactor 2 <sup>c</sup>				
		24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
Raf	4.55	4.35	4.29	4.35	4.42	4.29	3.40	3.35	3.40	3.45	3.35
Suc	92.65	91.12	106.28	106.56	113.69	100.55	93.75	87.90	87.30	84.90	61.30
Glu	1.30	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00
Gal	0.50	0.82	0.91	0.86	0.89	0.82	1.30	1.30	1.35	1.30	1.25
Fru	3.55	0.64	0.82	0.56	0.65	2.06	0.95	0.55	0.40	0.60	0.85
Gly	0.45	0.98	1.62	0.80	0.27	1.00	1.30	1.05	0.40	0.70	2.65
Eth	0.00	5.34	7.98	0.00	0.00	5.19	6.60	13.20	0.00	4.30	10.50
Sor	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

<sup>a</sup>Concentrations from 4 to 0.06 mg/mL of a mixture of raffinose (Raf), sucrose (Suc), galactose (Gal), glucose (Glu), fructose (Fru), glycerol (Gly), and ethanol (Eth) were used as external standard. Both samples and external standard were added with 1 mg/mL sorbitol (Sor) used as an internal standard, N = 3 ± DE.

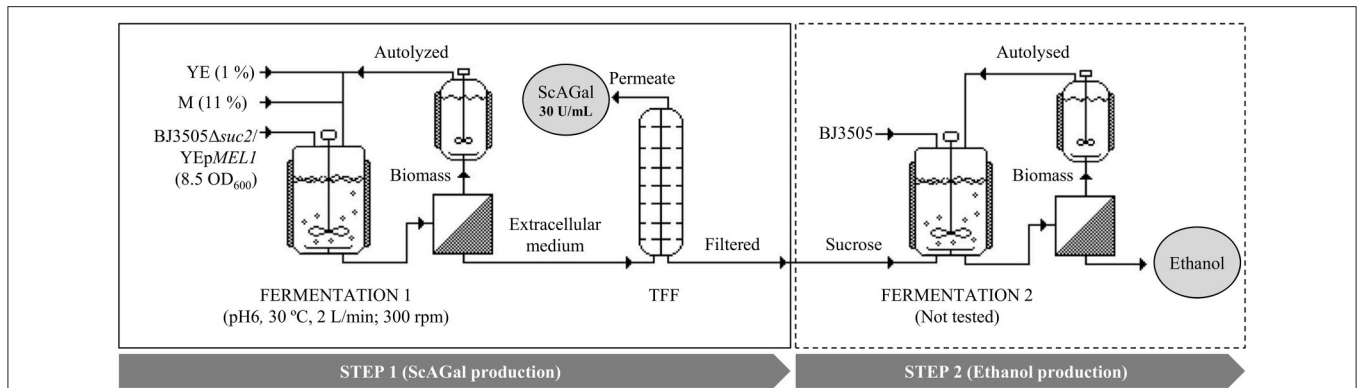
<sup>b</sup>Bioreactor 1 was carried out without adjusted pH during the culture course.

<sup>c</sup>Bioreactor 2 was maintained at pH 6 during the culture course.

et al., 2007). The accumulation of trehalose, as osmotic protector, with similar retention time than sucrose or the induction of the synthesis of other hydrolases involved in sucrose metabolism (Marques et al., 2015) could explain the increase and decrease in sucrose levels observed during cultures without and with pH control, respectively (Table 5).

Finally, we reported for the first time the statistical optimization of the production of ScAGal from molasses (using CCD) with high yields of enzyme production, while

the fermentation to ethanol of the remaining sucrose was just verified but not optimized since this procedure is nowadays ongoing at industrial scale. In addition, the stability of the plasmid expressing ScAGal is not a problem since 98% of the cells growing in molasses retained the plasmid at the end of the cultures. In fact, we obtained a production of 30 U/mL of ScAGal which is 1.6-fold higher than the recently reported with a *Kluyveromyces lactis* engineered strain growing in a mixture of cheese whey and molasses (Álvarez-Cao et al., 2018) and higher



**FIGURE 10 |** Simulation of a two-step bioprocess to obtain ScAGal and ethanol from beet molasses-based medium. The ended-culture of the fermentation 1 carried out by the invertase-deficient strain BJ3505 $\Delta$ suc2/YEpMEL1 is centrifuged, and the extracellular medium filtered by TFF to obtain a permeate with the recombinant protein, ScAGal (Step 1). The sucrose recovered in the filtrate is used in the fermentation 2 to produce ethanol by a strain of *S. cerevisiae* with invertase activity (Step 2). The biomass recovered from both fermentations can be used as yeast extract (YE) supplement after an autolysis treatment. M, beet molasses; YE, yeast extract; TFF, tangential flow filtration. Modified imagen from SuperPro Designer.

than the 10.4 U/mL obtained for *Aspergillus fumigatus* alpha-galactosidase expressed in *A. sojae* in an optimized synthetic medium in the presence of 10.5% (w/v) molasses (Gurkok et al., 2011). The alpha-galactosidase yield reached is among the highest of those obtained in submerged fermentation for the alpha-galactosidase enzyme of different origins on various carbon sources (Anisha, 2017).

## CONCLUSION

A procedure for valuation of beet molasses has been developed that consists in the high-yield production of ScAGal, an enzyme with wide market, by an engineered yeast strain from the less abundant sugars, followed by ethanol fermentation of sucrose, the most abundant sugar, by a wild type strain. The engineered strain is a  $\Delta$ suc2 mutant transformed with a plasmid that drives secretion of ScAGal to the culture medium. The mutation causes a shift to a more respiratory metabolism in molasses and facilitates extracellular ScAGal purification by avoiding contamination with invertase.

## DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

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## AUTHOR CONTRIBUTIONS

M-EC, M-IG-S, and MB contributed conception and design of the study. M-EA-C performed the experimental work (data acquisition); M-EA-C, M-IG-S, and MB contributed to analysis or interpretation of data. M-EA-C and M-IG-S wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00405/full#supplementary-material>

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# A Comprehensive Analysis of the Small GTPases Ypt7 Involved in the Regulation of Fungal Development and Secondary Metabolism in *Monascus ruber* M7

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Ypts (yeast protein transports), also called as *ras*-associated binding GTPases (Rab), are the largest group of the small GTPases family, which have been extensively studied in model eukaryotic cells and play a pivotal role in membrane trafficking, while this study showed potential regulation role of Ypts in fungi. One of Ypts, Ypt7 may be involved in fungal development and secondary metabolism, but the exact mechanism still exists a controversy. In current study, the functions of a *Monascus ypt7* homologous gene (*mrypt7*) from *Monascus ruber* M7 was investigated by combination of gene-deletion ( $\Delta mrypt7$ ), overexpression (*M7::PtrpC-mrypt7*) and transcriptome analysis. Results showed that the radial growth rate of  $\Delta mrypt7$  was significantly slower than *M. ruber* M7, little conidia and ascospores can be observed in  $\Delta mrypt7$ , but the yield of intracellular secondary metabolites was dramatically increased. Simultaneously, the *mrypt7* overexpression strain possessed similar capacity for sporulation and secondary metabolism observed in *M. ruber* M7. Transcriptome results further illustrated that *mrypt7* could coordinate with numerous genes involved in the vegetative growth, conidiogenesis, secondary metabolism biosynthesis and transportation of *M. ruber* M7. Combined with the similar effect of Ypt7 homologs on other fungi, we propose that Ypt7 works more like a global regulatory factor in fungi. To our knowledge, it is the first time to investigate Ypt7 functions in *Monascus*. It could also improve the understanding of Ypt7 functions in fungi.

**Keywords:** Ypt7, *Monascus*, development, secondary metabolism, regulation

## INTRODUCTION

The largest subfamily of *rat sarcoma* (Ras) superfamily, *ras*-associated binding GTPases (Rab), also called as Ypt (yeast protein transport) and Sec (*secretion*) (Gallwitz et al., 1983; Salminen and Novick, 1987), are involved in the membrane trafficking regulation in all eukaryotes (Maringer et al., 2016; Shinde and Maddika, 2016; Yun et al., 2016; Pfeffer, 2017). As key regulators of membrane transportation, Rab GTPases cycle between GTP-bound (active) and GDP-bound (inactive) conformations which stimulated by guanine nucleotide exchange factors (GEFs). Typical



Rab GTPase possess several conserved functional regions, including phosphate/Mg<sup>2+</sup> binding domain (PM), GTP/GDP binding domain (G), C-terminal isoprenylation region (C), and so on. For example, the G domain provides phosphate contacts and supplies a Ser/Thr site which is co-ordinated by the Mg<sup>2+</sup> ion. The conserved molecular switch mechanism have detailed in some reviews (Lee et al., 2009; Pylypenko et al., 2018). The first Ypt was discovered in *Saccharomyces cerevisiae* (Schmitt et al., 1986; Pereira-Leal, 2008; Li and Marlin, 2015), and the succedent research results have showed that there are total 11 encoded Ypt proteins in *S. cerevisiae*, and each of which possesses distinctive function at a particular stage of the membrane transport pathway (Pereira-Leal, 2008; Li and Marlin, 2015). In animal, dozens of Rab/Ypt are proven to regulate vesicle trafficking among organelles (Ohbayashi and Fukuda, 2012; Li and Marlin, 2015; Mignogna and D'Adamo, 2017; Pfeffer, 2017). In plant, Ypts also are required for intracellular trafficking from the trans-Golgi-network to the plasma membrane and/or prevacuolar compartments (Yun et al., 2016; Tripathy et al., 2017). The more detailed Ypts roles for vesicle transports in animal and plant are summarized in the previous reviews (Stenmark, 2009; Ao et al., 2014).

In fungi, the number of Ypt family is stable from 7 to 12 Ypts, each of which may be responsible for a particular stage of the membrane transport pathway (Pereira-Leal, 2008; Li and Marlin, 2015). Among them, Ypt7 is proved as a key regulator of the material movement and transformation among cellular compartments through vacuolar biogenesis and fusion (Ohsumi et al., 2002; Kashiwazaki et al., 2009; Balderhaar et al., 2010; Wickner, 2010), and the Ypt7-mediated vacuolar fission and fusion are proved to be essential for maintaining stabilities of the cytosolic pH and osmolarity, and storing and transferring intermediary metabolites like mammalian lysosomes and plant vacuoles (Richards et al., 2010; de Marcos Lousa and Denecke, 2016; BasuRay et al., 2018), while some investigations have also showed that Ypt7 can influence fungal development and secondary metabolism. For example, the *ypt7* gene deletion or overexpression can lead to the variances of conidiogenesis and metabolism in fungi (Chanda et al., 2009a; Xu et al., 2012; Li et al., 2015; Liu et al., 2015; Zheng et al., 2015). However, it is still unclear how Ypt7 regulates fungal development and secondary metabolism, and the relationship among Ypt7-mediated vacuolar changes and fungal development and secondary metabolism.

*Monascus* spp., as one of the important edible filamentous fungi, can produce many beneficial secondary metabolites (SMs) including *Monascus* pigments (Mps), monacolin K (MK),  $\gamma$ -aminobutyric acid and so on (Patakova, 2013; Wu et al., 2013). As such, its fermented products, red yeast rice, also named as *Hongqu* in China have been used as food additives for more than 2,000 years (Chen et al., 2015). What's more, *Hongqu* has been permitted to use as a food supplement in USA from 1900s due to its cholesterol-lowering effects (Heber et al., 1999). The European Food Safety Authority (EFSA) also published a scientific opinion related to the daily dose of *Hongqu* containing MK (EFSA, 2011). Although citrinin (CIT), a nephrotoxic toxin produced by some *Monascus* strains ever hampered *Hongqu* use, nowadays the control and elimination of CIT in *Hongqu* have successfully been

solved by the strain screenings or molecular biological techniques (Shimizu et al., 2005; He and Cox, 2016).

There were 7 *ypt* homologous genes (*ypt1-ypt7*), which functions are predicted (Table S1), have been discovered in the genome of *Monascus ruber* M7. In current paper, the functions of *Monascus ypt7* (*mrypt7*) gene were investigated by combination of gene disruption, overexpression and transcriptome analysis. The results have revealed that besides the membrane trafficking regulation like other fungi, *mrypt7* can also coordinate with numerous genes involved in the development and metabolism of *M. ruber* M7. Combined with Ypt7 functions in other fungi (Bouchez et al., 2015; Liu et al., 2015; Yang et al., 2018), we discuss and propose that Ypt7 works more like a global regulatory factor in fungi. To our knowledge, it is the first time to investigate Ypt7 functions in *Monascus* which could help us to improve the understanding of Ypt7 functions in fungi.

## MATERIALS AND METHODS

### Fungal Strains, Culture Media, and Growth Conditions

*M. ruber* M7 (CCAM 070120, Culture Collection of State Key Laboratory of Agricultural Microbiology, Wuhan, China), which can produce Mps and CIT, but no MK (Chen and Hu, 2005; Chen et al., 2017), was used to generate the *mrypt7* deletion strain ( $\Delta$ *mrypt7*) and overexpression strain (M7::*PtpC-mrypt7*). The potato dextrose agar medium (PDA), Czapek yeast extract agar medium (CYA), glycerol nitrate agar medium (25%) (G25N) and malt extract agar medium (MA) were utilized to observe the strains phenotypic characterization (He et al., 2013). Neomycin-resistant transformants were selected on PDA media containing 15  $\mu$ g/mL G418 (Sigma-Aldrich, Shanghai, China). All strains were maintained on PDA slant at 28°C.

### Cloning and Sequence Analysis of *mrypt7* in *M. ruber* M7

Ypt family genes in *M. ruber* M7 genome were blast from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table S1). Amino acid sequence encoded by *mrypt7* was predicted using the SoftBerry's FGENESH program (<http://linux1.softberry.com/>). *mrypt7* homology was compared with 283 fungi Ypt7 downloaded from NCBI to analyze their primary structural features (<http://weblog.threeplusone.com/create.cgi>).

### Construction and Verification of *mrypt7* Gene Deletion and Overexpression Strains

The construction and verification of *mrypt7* gene deletion and overexpression strains were implemented according to the literature references (Shao et al., 2009; Liu et al., 2014). Briefly, the *mrypt7* gene deletion cassette (5' UTR-G418-3' UTR) and *mrypt7* gene overexpression cassette (5' UTR-G418-*PtpC-mrypt7*-3' UTR) were constructed by double-joint PCR with the primers listed in Table S2 (Yu et al., 2004), and shown schematically in Figure S1. The *mrypt7* gene deletion and overexpression vectors were formed, and transformed to *M. ruber* M7 using *Agrobacterium tumefaciens*-mediated transformation system

to generate the *mrypt7* gene deletion mutants ( $\Delta mrypt7$ ) and overexpression transformants (*M7::PtrpC-mrypt7*), respectively. PCR and southern blot were used to verify the *mrypt7* gene deletion and overexpression strains.

## Phenotypic Characterization

*M. ruber* M7,  $\Delta mrypt7$  and *M7::PtrpC-mrypt7* were cultivated on PDA, CYA, MA and G25N for 10 d at 28°C to observe phenotypic characterization. Besides, the three above-mentioned strains were incubated on PDA for 3 d at 28°C for vacuole morphological observation. For a better distinction, the normal vacuoles were designated vacuoles (Va), while smaller vacuoles were designated fragment vacuoles (Fv) (Chanda et al., 2009b).

## Detection of Mps and CIT

One milliliter freshly harvested spores ( $10^5$  cfu/mL) of each strain were inoculated on PDA plate covered with cellophane membranes, and incubated at 28°C for 11 days, the samples were taken every 2 days from the 3rd day to the 11th day of culture to measure the Mps and CIT production. Freeze-dried mycelia and medium powder (0.1 g) was suspended in 1 mL 80% (v/v) methanol solution, and subjected to 30 min ultrasonication treatment (KQ-250B, Kunshan, China).

The Mps and CIT were separated by an ACQUITY UPLC BEH C18 column (2.1 mm  $\times$  100 mm, 1.7  $\mu$ m), and detected on Waters ACQUITY UPLC I-class system (Waters, Milford, MA, USA). A gradient elution was performed with the mobile phase including solvent A (0.1% formic acid in water) and solvent B (acetonitrile) with a flow rate of 0.3 mL/min and an injection volume of 2  $\mu$ L. The gradient elution was performed as follows: 60% (v/v) solvent A with 40% (v/v) solvent B maintained for 0.5 min firstly, the content of solvent A was decreased from 60 to 20% for 21 min, and then from 20 to 60% for 0.5 min. Finally, the column was equilibrated with 60% solvent A for 3 min. The temperature of chromatographic column and samples were maintained at 40°C and 4°C, respectively.

## RNA Extraction, Library Preparation and Sequencing

Since *M. ruber* M7 and *M7::PtrpC-mrypt7* shared similar phenotype and SMs yield (Figures 2, 3), the *mrypt7* functions were further investigated only between *M. ruber* M7 and  $\Delta mrypt7$  by transcriptome analysis. Detailly, 1 mL freshly harvested spores ( $10^5$  cfu/mL) of *M. ruber* M7 and  $\Delta mrypt7$  were inoculated on PDA plate covered with cellophane membranes, and incubated at 28°C. Besides, based on our previous results, *Monascus ruber* M7 starts conidiation at 3rd day on PDA medium, and the secondary metabolized yield reached a relatively high level in 7th day, so the mycelium after cultured 3 days and 7 days were collected and used for the total RNA extraction by TRIzol Reagent (Invitrogen, Life Technologies, USA), two biological replicates were designed for each condition (Muraguchi et al., 2015; Srikumar et al., 2015; Heuston et al., 2018). The RNA purity and integrity were analyzed by Nanodrop NanoPhotometer spectrophotometer (NanoDrop products IMPLN, CA, USA) and Agilent 2100 BioAnalyzer (Agilent, USA).

For each sample, the cDNA library was constructed using RNA Library Prep Kit for Illumina (NEB, USA). The obtained PCR products were purified by AMPure XP system and library quality was assessed on the Agilent Bioanalyzer 2100 system. The eight samples (*M7-3d* vs. *M7-7d*,  $\Delta mrypt7$ -3d vs.  $\Delta mrypt7$ -7d, *M7-3d* vs.  $\Delta mrypt7$ -3d and *M7-7d* vs.  $\Delta mrypt7$ -7d, with two repeats in each group) were sequenced using the BGISEQ-500RS platform (BGI, Wuhan, China, <http://www.mgitech.cn/product/detail/BGISEQ-500.html>).

## Sequence Quality Evaluation and Validation

The obtained sequence raw reads of above-mentioned 8 samples were saved as FASTQ files, then the clean data were obtained after removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. The expression levels of 10 randomly selected genes in *M. ruber* M7 were validated by qRT-PCR following the protocol of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Japan) and the SYBR<sup>®</sup> Select Master Mix (ABI, USA).

## Functional Analysis of Transcriptome Data

The *M. ruber* M7 genome which contains 8,407 genes was used as a reference genome (Chen, 2015) to calculate the blast rate of genome and clean data by Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT) and Bowtie2 (Langmead and Salzberg, 2012; Kim et al., 2015).

Gene expression levels were estimated by RNA-Seq by Expectation-Maximization (RSEM), the normalized value of fragments per kilobase of transcript per million mapped reads (FPKM) was used as a parameter to compare the expression levels between *M. ruber* M7 and  $\Delta mrypt7$  (Li and Dewey, 2011; Van Verk et al., 2013). The orthologs with significantly different expression were identified by NOISeq method with an absolute value of  $\log_2$ -fold change >1 and probability >0.8 (Tarazona et al., 2012).

Gene ontology (GO) (<http://www.geneontology.org/>) and KEGG pathway (<http://www.kegg.jp/>) function analysis were implemented to investigate the functions of the differentially expressed genes (DEGs) between *M. ruber* M7 and  $\Delta mrypt7$ . Moreover, the DEGs involved in fungal growth, sporation and secondary metabolism were further analyzed to illuminate the *Mrypt7* role in fungal development and secondary metabolism.

## RESULTS

### Sequence Analysis and Characterization of *mrypt7* in *M. ruber* M7

The Ypt family genes in *M. ruber* M7 genome were blasted from NCBI, totally 7 Ypts showed highly homologous with other fungi (Table S1). Among them, Ypt7 homology was further analyzed in this study. Detailly, a 954 bp fragment containing the putative *mrypt7* homolog was successfully amplified from the genomic DNA of *M. ruber* M7. A database searched with softberry (<http://linux1.softberry.com/berry.phtml>) has been showed that the CDS (coding sequence) length of *mrypt7* gene is 591 bp which encodes 196-amino acids and consists of 5 exons

(**Figure S2**). The characteristic motifs or residues of Ypt7 from M7 and other 283 fungi downloaded from NCBI were investigated, and the results illustrated that phosphate/Mg<sup>2+</sup> binding domain (PM), GTP/GDP binding domains (G) and C-terminal isoprenylation region (C) are highly conserved in all tested fungi (**Figure S2**). Besides, a database searched with NCBI-BLAST has been demonstrated that the deduced 196-amino acid sequences encoded by *mrypt7* share 91% similarity with the GTP-binding protein Ypt7 of *Aspergillus fischeri* (Genbank: XP\_001259484.1), *A. oryzae* (Genbank: XP\_001824054.1), *A. niger* (Genbank: XP\_001398680.2), *P. oxalicum* (Genbank: EPS32522.1), and *P. zonata* (Genbank: XP\_022585464.1) (**Table S1**).

## Verification of the *mrypt7* Deletion and Overexpression Strains

Total 9 putative disruptants ( $\Delta$ *mrypt7*) were obtained and analyzed, and one of them was displayed here. In PCR analysis as shown in **Figure 1A**, no DNA band was amplified when the genome of the putative  $\Delta$ *mrypt7* strain was used as template with the primer pair Y7-up1/Y7-do1 (**Table S1**), while a 0.7 kb product appeared using the genome of the wild-type strain *M. ruber* M7. A 1.2-kb fragment of *G418* gene could be amplified from  $\Delta$ *mrypt7* using primers G418up/G418do (**Table S1**), while nothing could be obtained from *M. ruber* M7. Meanwhile, amplicons of *M. ruber* M7 (2.3 kb) and  $\Delta$ *mrypt7* (2.4 kb) different in size were observed when primers Y7-Zup1/Y7-Ydo1 (**Table S1**) were used.

The putative  $\Delta$ *mrypt7* was further verified by Southern blot. As showed in **Figure 1B**, a probe corresponding to the *mrypt7* coding region (probe 1, **Table S2**) yielded a single hybridizing band in a Southern blot of *Hind*III-digested genomic DNA of the wild-type strain, compared with no band in  $\Delta$ *mrypt7*, which demonstrated that *M. ruber* M7 carried a single copy of *mrypt7*. Meanwhile, no band was detected in the wild-type strain, while a single band occurred in  $\Delta$ *mrypt7* using probe 2 (**Table S2**) which corresponds to the *G418* gene. These results proved that  $\Delta$ *mrypt7* carried a single integrated copy of the *mrypt7* disruption cassette.

Total 16 putative *M7::PtrpC-mrypt7* strains with *G418* resistance were obtained and analyzed, and one of them was showed as follows. In PCR analysis as shown in **Figure 1C**, a 1.2-kb product appeared when the genome of the putative *M7::PtrpC-mrypt7* strain was used as template with primers G418up/G418do (**Table S2**), while no DNA band was amplified using the genome of *M. ruber* M7. Amplicons of *M. ruber* M7 (3.0-kb) and *M7::PtrpC-mrypt7* (4.5 kb and 3.0 kb) were totally different in size when primers Y7-up1/Y7-do1 (**Table S2**) was used, which proved that there were two copies of the *mrypt7* overexpression cassette integrated in *M7::PtrpC-mrypt7*.

Southern blot analysis (**Figure 1D**) showed that probe 1 (**Table S2**) yielded two bands in *M7::PtrpC-mrypt7* and a single band in *M. ruber* M7, while

probe 2 (**Table S2**) generated a single band in *M7::PtrpC-mrypt7* and no band in *M. ruber* M7, which demonstrated that *M7::PtrpC-mrypt7* carried two integrated copies of the *mrypt7* and was a successful homologous recombination event.

qRT-PCR was implemented to analyze the transcription levels of the *mrypt7* gene in *M. ruber* M7,  $\Delta$ *mrypt7* and *M7::PtrpC-mrypt7*. As shown in **Figure 1E**,  $\Delta$ *mrypt7* was deficient in the expression of the *mrypt7* gene, the average level of *mrypt7* expression in *M7::PtrpC-mrypt7* was five times higher than that of *M. ruber* M7. These results further verified the success of gene knockout and overexpression in the putative  $\Delta$ *mrypt7* and *M7::PtrpC-mrypt7* strains.

## Phenotypic Characterization of $\Delta$ *mrypt7*, *M7::PtrpC-mrypt7* and *M. ruber* M7

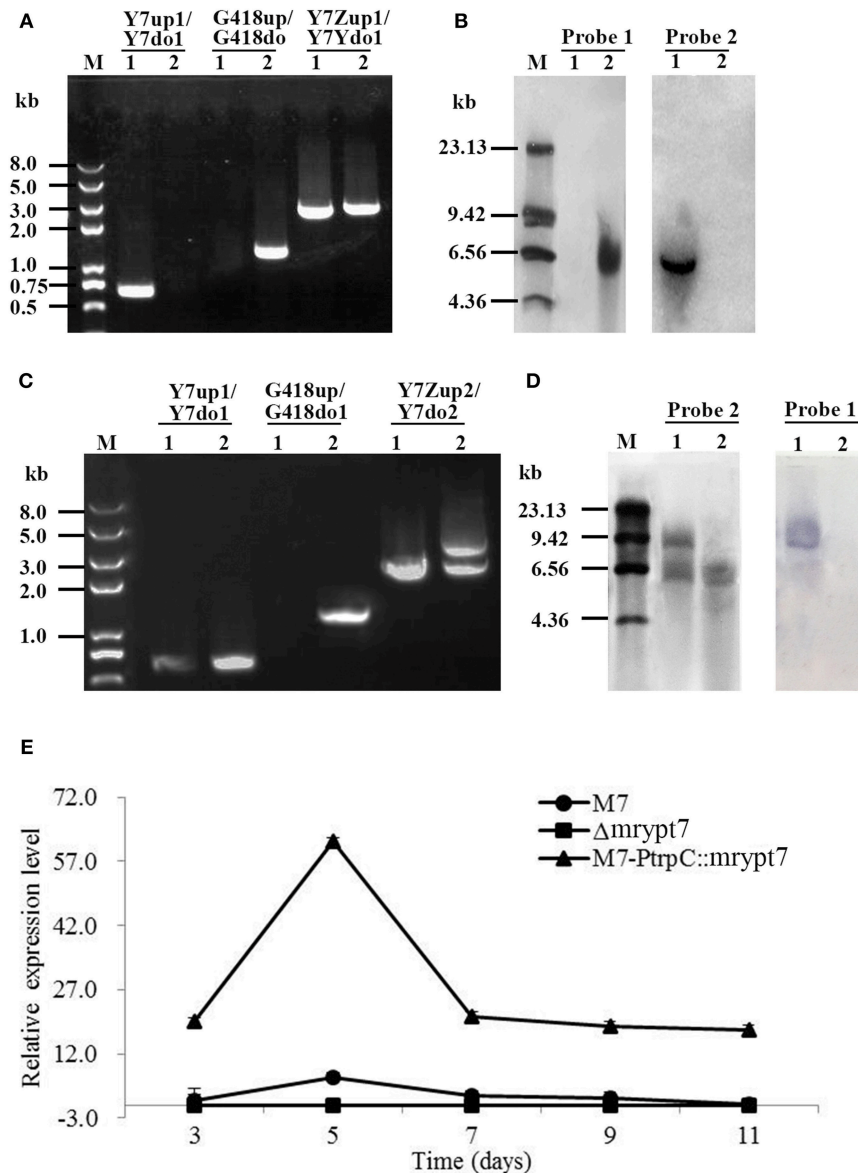
Phenotypes of *Monascus ruber* were observed on the different media (PDA, CYA, MA, G25N) to investigate the influences of the *mrypt7* deletion and overexpression on developmental processes. As showed in **Figure 2A**, the colony edge of  $\Delta$ *mrypt7* was irregular and the growth rates of  $\Delta$ *mrypt7* was slower than those of *M7::PtrpC-mrypt7* and *M. ruber* M7. Besides, cleistothecia and conidia formation of  $\Delta$ *mrypt7* were obviously inhibited compared with *M7::PtrpC-mrypt7* and *M. ruber* M7. While the colony phenotypes, growth rates and conidia formation of *M7::PtrpC-mrypt7* had no significantly difference from those of *M. ruber* M7 (**Figure 2B**).

Vacuoles (Va) and fragment vacuoles (Fv) of *M. ruber* M7,  $\Delta$ *mrypt7* and *M7::PtrpC-mrypt7* on PDA medium were also observed under microscope. Compared with *M7::PtrpC-mrypt7* and *M. ruber* M7, the number of Fv in  $\Delta$ *mrypt7* increased more, while vacuoles reduced relatively and distributed nonuniformly in the mycelia (**Figure 2C**). The Fv and Va number and distribution between *M7::PtrpC-mrypt7* and *M. ruber* M7 had no big difference, but the more uniform Fv and Va distribution of *M7::PtrpC-mrypt7* was apparent (**Figure 2C**).

## Mps and CIT Production Analysis of $\Delta$ *mrypt7*, *M7::PtrpC-mrypt7* and *M. ruber* M7

Previous studies (Chen et al., 2017) have demonstrated that *M. ruber* M7 can produce Mps and CIT, but no MK, so the yields of the 8 main Mps (four yellow pigments, monasfloure A, monascine, monasfloure B, ankaflavin; two orange pigments, rubropunctatin, monascuburin; two red pigments, rubropunctamine and monascuburamine) and CIT in *M. ruber* M7 and its mutants were analyzed in this study to uncover the effect of *Mrypt7* on SMs. Generally, all the detected SMs were increased in the mycelia of  $\Delta$ *mrypt7* and *M7::PtrpC-mrypt7*, compared to *M. ruber* M7 (**Figures S3, S4**). Take monasfloure A, rubropunctatin, rubropunctamine and CIT production as examples for detail explanation, as showed in **Figure 3**, the concentration of intracellular yellow, orange and





**FIGURE 1 |** The verification of *mrypt7* deletion and overexpression mutants. **(A)** PCR verification of the *mrypt7* deletion mutant; **(B)** Southern blot verification of the *mrypt7* deletion mutant; **(C)** PCR verification of the *mrypt7* overexpression mutant; **(D)** Southern blot verification of the *mrypt7* overexpression mutant; **(E)** qRT-PCR analysis of *mrypt7* gene expression level.

red pigments in  $\Delta$ *mrypt7* were 1.8 times, 1.3 times, and 2.8 times of those in *M. ruber* M7, respectively, while the production of extracellular yellow, orange and red pigments were 63, 45, and 83% of *M. ruber* M7. In contrast, both intracellular and extracellular Mps in M7::PtrpC-*mrypt7* were increased at least 20% compared with *M. ruber* M7. The intracellular CIT concentration in  $\Delta$ *mrypt7* in 11th day was nearly 5 times more than those in M7::PtrpC-*mrypt7* and *M. ruber* M7, while the extracellular CIT was only 20~40% of that in M7::PtrpC-*mrypt7* and *M. ruber* M7. The intracellular and extracellular CIT in M7::PtrpC-*mrypt7* and *M. ruber* M7 possessed the similar concentration.

### The Mrypt7 Function Elucidation on Development and Secondary Metabolite Production by Transcriptome Analysis Differentially Expressed Genes Analysis, Annotation and Functional Classification

The transcriptome data obtained by RNA-seq were validated by qRT-PCR,  $\beta$ -actin serving as the reference gene. The expression data of 10 randomly selected genes (GME3693, GME5196, GME5065, GME2292, GME2157, GME5531, GME67, GME3412, GME6749, GME4561, GME2587) which are from the genome of *M. ruber* M7, fit with the sequencing profiles (**Figure S5**).



The differentially expressed genes (DEGs) between M7-3d vs. M7-7d,  $\Delta mrypt7$ -3d vs.  $\Delta mrypt7$ -7d, M7-3d vs.  $\Delta mrypt7$ -3d and M7-7d vs.  $\Delta mrypt7$ -7d were analyzed. The DEGs' functions were analyzed through GO function classifications and KEGG pathway. According to GO categories, the DEGs function classifications of the four teams almost belong to biological process, cellular component and molecular function. KEGG pathway analysis manifested that the DEGs were mostly involved in cellular process, environmental information processing, genetic information processing, human diseases and metabolism. For example, the DEGs down-regulated in M7-3d vs.  $\Delta mrypt7$ -3d included ankyrin repeat protein, G protein-coupled receptor and thiazole synthase, meanwhile, the DEGs up-regulated in M7-3d vs.  $\Delta mrypt7$ -3d included syntaxin, Golgi SNAP receptor, Ras GTPase activating like protein and fungal type III polyketide synthase. The DEGs down-regulated in M7-7d vs.  $\Delta mrypt7$ -7d included acyl-CoA synthetase, transposon, ubiquinone biosynthesis protein, exosome complex component and regulator of ribosome biosynthesis; while the DEGs up-regulated in M7-7d vs.  $\Delta mrypt7$ -7d included golgi family apparatus membrane protein, mitochondrial fission protein, vesicular inhibitory amino acid molecule and gamma tubulin complex.

### The Fungus-Specific Regulators Coordinating Conidia Were Positively Regulated by Mrypt7

Fungal conidiation regulatory mechanism is very complex, and there are many regulators involved in fungal conidiation which can be divided into central regulators (*brlA*, *abaA*, and *wetA*), upstream activators (*fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*), negative regulators (*CpcB*, *NsdC*, *NsdD*, *OsaA*, *SfgA*, and *VosA*, etc.), velvet regulators (*VeA*, *VelB*, *VelC*, and *VosA*) and light responsive regulators (*FphA*, *CryA*, *LreA*, and *LreB*) (Park and Yu, 2012, 2016). The putative regulatory DEGs coordinating conidiation in M7 and  $\Delta mrypt7$  were analyzed in this part to illustrate the regulation of *mrypt7* on *Monascus* conidia (Table 1).

The central regulatory pathway controls conidiation-specific gene expression and asexual developmental processes, very interesting, in *M. ruber* M7, the central regulatory pathway only includes *brlA* and *wetA* without *abaA* (Chen, 2015). Transcriptome results showed *brlA* and *wetA* were significantly down-regulated in  $\Delta mrypt7$ -3d vs. M7-3d, even the *brlA* gene was up-regulated in  $\Delta mrypt7$ -7d vs. M7-7d, the total expression levels of *brlA* in  $\Delta mrypt7$ -3d and  $\Delta mrypt7$ -7d were lower than those in *M. ruber* M7. Besides, the *flbD* gene belongs to one of the upstream developmental activators which is required for the initiation of conidiation and *brlA* activation (Kwon et al., 2010) was down-regulated in  $\Delta mrypt7$ -7d. For balancing with upstream activators, on the contrast, the *cpcB* gene belongs to the negative regulator which inhibits precocious activation of *brlA* during proliferation (Park and Yu, 2012) was up-regulated in  $\Delta mrypt7$ -3d. What's interesting is that the velvet regulators, *veA* and *velB* which suppresses conidiation and activation of sexual development (Bayram and Braus, 2012; Park and Yu, 2016) were up-regulated in  $\Delta mrypt7$ -7d, but little cleistothecia could be found in  $\Delta mrypt7$  (Figure 2B), which was different from the results found in *Aspergillus nidulans* (Kim et al., 2002).

### Effect of Mrypt7 on the Secondary Metabolites Biosynthesis Process

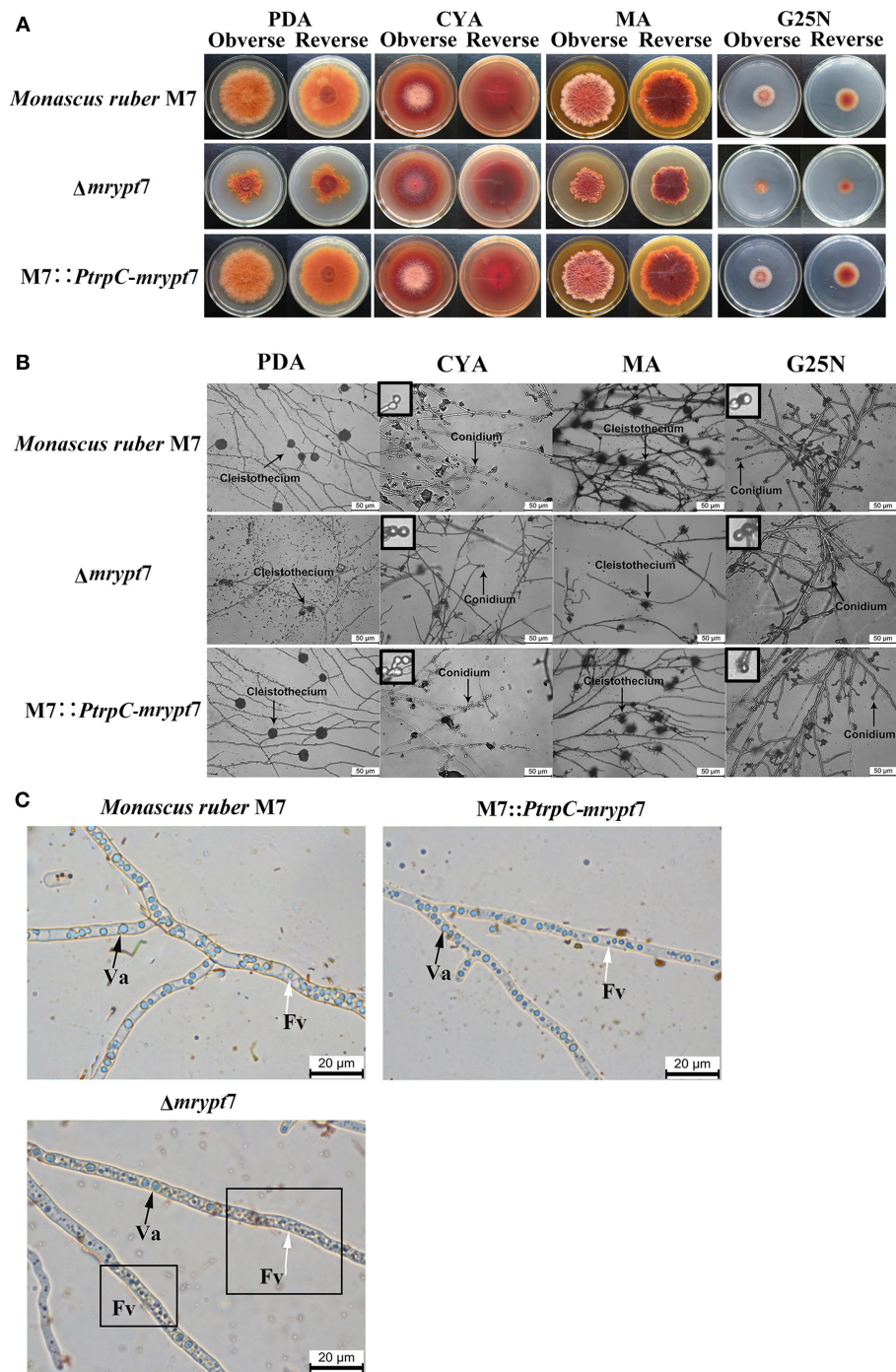
*Monascus* spp. can produce several secondary metabolites, like Mps, CIT, and so on (Liao et al., 2014; Feng et al., 2016). Previous studies have demonstrated that there are 9 predicted *pks* (polyketone synthase) genes in *M. ruber* M7 genome (Chen, 2015), and the different effects of Mrypt7 on these 9 *pks* genes were listed in Table 2. Among them, only the Mps *pks* was down-regulated in M7-3d vs. M7-7d, while all *pks* genes were up-regulated in  $\Delta mrypt7$ -3d vs.  $\Delta mrypt7$ -7d even only conidial yellow pigment *pks* and Mps *pks* reaching the significantly difference levels ( $\log_2$ -fold change > 1 and probability > 0.8). Besides, all *pks* genes down-regulated in M7-3d vs.  $\Delta mrypt7$ -3d and only Mps *pks* gene and a putative lovastatin nonaketide *pks* gene reaching the significantly difference levels; while the putative lovastatin nonaketide synthase down-regulated and conidial yellow pigment, CIT and Mps *pks* up-regulated in M7-7d vs.  $\Delta mrypt7$ -7d. Combination of these results has revealed that Mrypt7 can remarkably affect the expression of genes involved in SMs biosynthesis, but Mps and CIT *pks* genes may be more affected by Mrypt7.

Further analysis of the expression level of Mps and CIT biosynthesis gene clusters showed that most of these genes down-regulated in M7-3d vs.  $\Delta mrypt7$ -3d and up-regulated in M7-7d vs.  $\Delta mrypt7$ -7d. Generally, for Mps biosynthesis gene cluster (Chen et al., 2017), the expressions of all genes (except *MpigH*, *MpigI*, and *MpigL*) were down-regulated in M7-3d vs. M7-7d, only *MpigH* and *MpigL* was up-regulated in  $\Delta mrypt7$ -3d vs.  $\Delta mrypt7$ -7d, the results suggested that the Mps biosynthesis gene cluster in  $\Delta mrypt7$  maintained a higher expression level compared with M7; nearly all genes down-regulated in  $\Delta mrypt7$ -3d vs. M7-3d but only *MpigA*, *MpigC*, *MpigE*, *MpigF*, *MpigH*, *MpigL*, and *MpigN* reaching the significantly difference levels, on the contrary, the whole Mps gene cluster (except *MpigH* and *MpigI*) were up-regulated in  $\Delta mrypt7$ -7d vs. M7-7d (Table 3).

While for the CIT biosynthesis gene cluster (He and Cox, 2016), the expressions of *pksCT*, *MRL7*, *MRL4*, *MRL2*, *MRL1*, *MRR2*, and *MRR2* down-regulated and *MRL5*, *MRR4* up-regulated in M7-3d vs. M7-7d; *MRL7*, *MRL6*, *MRL5*, *MRL4*, *MRL2*, *MRL1*, *pksCT*, and *MRR1* up-regulated and *MRR2*, *MRR5* down-regulated in  $\Delta mrypt7$ -3d vs.  $\Delta mrypt7$ -7d; only *MRL5* down-regulated and *MRR3* up-regulated in  $\Delta mrypt7$ -3d vs. M7-3d, but almost all genes (except *MRR5*) up-regulated in  $\Delta mrypt7$ -7d vs. M7-7d (Table 4).

## DISCUSSION

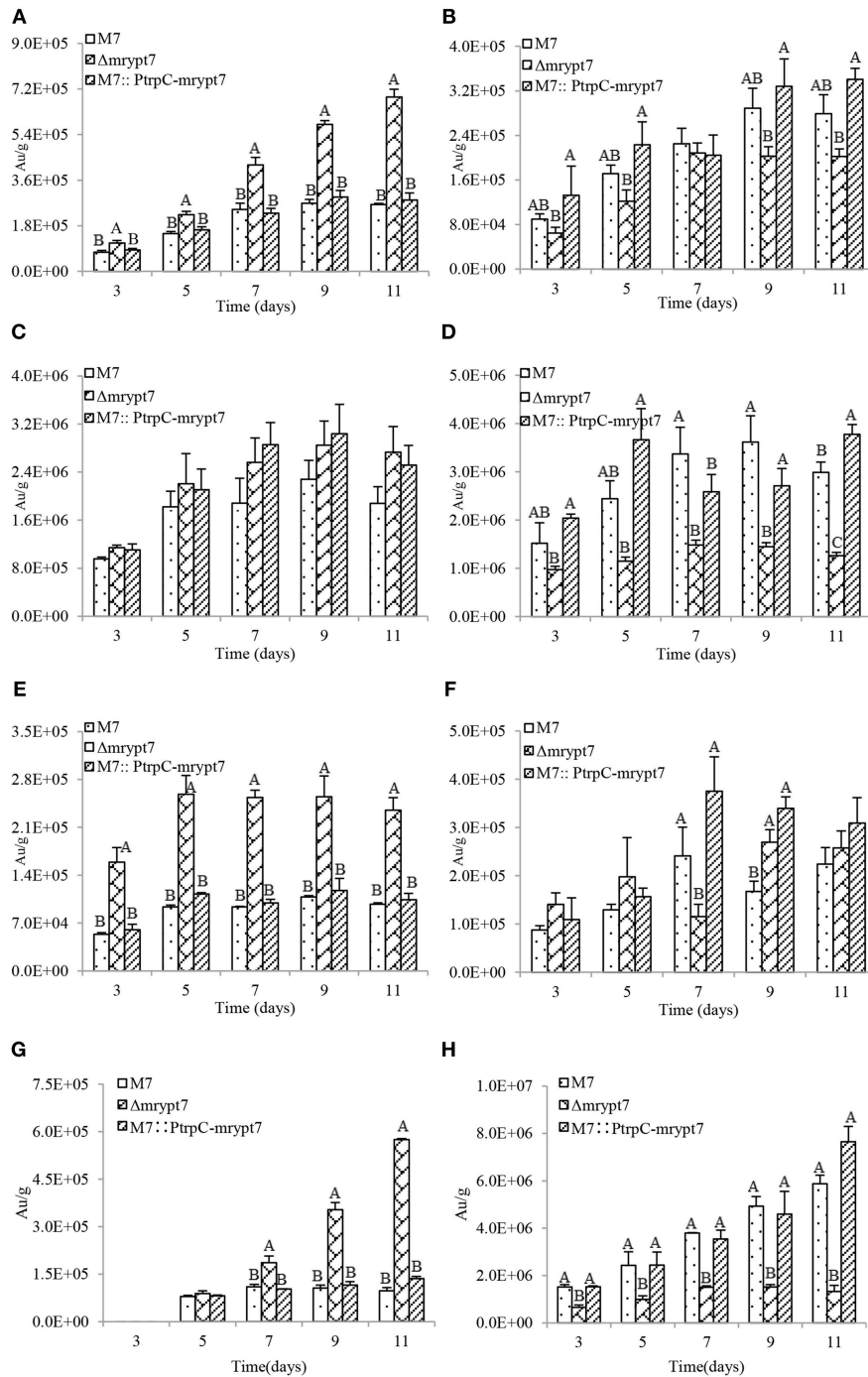
Ypt/Rab, a single-subunit small GTPase which is related in structure to the  $G\alpha$  subunit of heterotrimeric G proteins (large GTPases) (Santarpia et al., 2012), has been proved to be the key regulators of the membrane trafficking system, endocytosis and exocytosis in all eukaryotes, especially in animals and plants (Fu et al., 2017; Kim et al., 2017; Pfeiffer, 2017; Srikanth et al., 2017). In fungi, the functions of Ypt/Rab, especially



**FIGURE 2** | Phenotypic characterization of  $\Delta mryp7$ , M7::PtrpC-mryp7 and *M. ruber* M7. **(A)** Colony morphologies on PDA, CYA, MA and G25N plates; **(B)** Microscopic structures on PDA, CYA, MA, and G25N plates, the enlarged area was indicated by arrow, size bar = 50  $\mu$ m; **(C)** Vacuole and fragment vacuoles microscopic structures on PDA medium. Va, vacuole (black arrow); Fv, fragment vacuoles (white arrow).

Ypt7, also only focus on its role of vesicle transport. It's an accepted fact that Ypt7 mainly controls vesicle-vacuolar fusion balance, the disruption and overexpression of Ypt7 caused various vacuole phenotypes (Xu et al., 2012; Li et al., 2015; Liu et al., 2015; Zheng et al., 2015). Besides, the mechanism

of Ypt7 mediated fusion interacts with numerous tethering and SNARE (Soluble NSF attachment protein receptor) complexes had been proved (Balderhaar et al., 2010; Ng et al., 2012; Hyttinen et al., 2013). Moreover, conidiogenesis imperfection and SMs production variation can also be found in Ypt7



**FIGURE 3 |** Mps and CIT yield analysis of *M. ruber* M7,  $\Delta mrypt7$  and M7::PtrpC-mrypt7. **(A)** The yield of intracellular Monasfloure A. **(B)** The yield of extracellular Monasfloure A. **(C)** The yield of intracellular Rubropunctatin. **(D)** The yield of extracellular Rubropunctatin. **(E)** The yield of intracellular Rubropunctamine. **(F)** The yield of extracellular Rubropunctamine. **(G)** The yield of intracellular CIT. **(H)** The yield of extracellular CIT. The error bar represents the standard deviation between the three repeats. Capitals signify  $p$ -value  $< 0.01$ .

disruption or overexpression mutants (Chanda et al., 2009a; Li et al., 2015; Liu et al., 2015; Yang et al., 2018), but the mechanism of Ypt7 involved conidial biogenesis and SMS biosynthesis was unclear.

In current study, the functions of *mrypt7* (*ypt7* homologous) in *M. ruber* M7 were investigated, we have found that aside from the functions of vesicle fusion, Mrypt7 can synchronously regulate the vegetative growth, conidiogenesis

**TABLE 1** | The putative regulatory DEGs involved in growth and conidiation in *M. ruber* M7.

	Regulators	Gene accession	Means				Regulation*			
			M7-3d	M7-7d	$\Delta mrypt7$ -3d	$\Delta mrypt7$ -7d	M7-3d vs. M7-7d	$\Delta mrypt7$ -3d vs. $\Delta mrypt7$ -7d	M7-3d vs. $\Delta mrypt7$ -3d	M7-7d vs. $\Delta mrypt7$ -7d
Central regulators	<i>brlA</i>	GME2587	30.5	4.3	6.0	17.9	Down	Up	Down	Up
	<i>abaA</i>						/			
	<i>wetA</i>	GME2686	34.9	22.7	7.3	26.4	-	Up	Down	-
Upstream activators	<i>flbA</i>	GME7104	77.4	150.3	61.7	116.9	Up	-	-	-
	<i>flbD</i>	GME650	12.5	24.2	2.9	2.7	-	-	Down	Down
Negative activators	<i>fluG, flbB, flbC, flbE</i>						No significant difference			
	<i>cpcB</i>	GME2676	283.9	405.3	711.7	462.4	-	-	Up	-
	<i>fadA</i>	GME5261	110.0	188.7	84.2	166.6	-	Up	-	-
	<i>nsdC</i>	GME2944	96.8	184.4	96.9	191.2	Up	Up	-	-
	<i>nsdD</i>	GME7585	17.9	42.1	14.9	34.7	Up	Up	-	-
	<i>sfaD</i>	GME5747	69.8	151.8	77.8	114.7	Up	-	-	-
	<i>ganB, gpgA, osaA, sfgD</i>						No significant difference			
velvet regulators	<i>veA</i>	GME5196	78.3	52.2	83.8	162.2	-	Up	-	Up
	<i>velB</i>	GME7847	39.3	26.9	52.3	65.7	-	-	-	Up
	<i>velC</i>						/			
	<i>vosA</i>	GME6122	37.3	34.7	25.0	56.0	-	Up	-	-
Light responsive regulators	<i>fphA</i>	GME5823	0.2	0.1	0.3	0.1	-	-	-	-
	<i>lreA</i>						/			
	<i>lreB</i>						/			
	<i>cryA</i>						/			

\*Significantly different expression was identified by NOISeq method with an absolute value of  $\log_2$ -fold change > 1 and Probability > 0.8. "Up" means the gene was up-regulated in the sample set; "Down" means the gene was down-regulated in the sample set; "-" means the gene possessed similar expression level in the sample set; "/" means the gene had no homologs in *M. ruber* M7.

and secondary metabolism in *M. ruber* M7. Transcriptome results illustrated that the fungus-specific conidiation regulators and SMs biosynthesis genes expression were significantly difference when *ypt7* gene was deleted (Tables 1–4). So we propose that Ypt7 works more like a global regulatory factor in fungi, which is first put forward the novel function definition of Rab GTPases.

Fungal conidiation regulatory mechanism is very complex, and there are some differences for the regulatory gene distribution in different fungi. Compared to *A. nidulans*, the up-to-date regulatory genes were conserved in *M. ruber* M7, while no homolog hits of *abaA* (central regulators), *VelC* (velvet regulators), *CryA*, *LreA*, and *LreB* (light responsive regulators) were searched in *M. ruber* M7 (Table 1). It seems that a new regulatory network may be owned in *Monascus*. In current study, the *mrypt7* disruption repressed asexual development, meanwhile, the regulators (*brlA*, *wetA* and *flbD*) related to conidia were down-regulated (Figure 2, Table 1), similar results were also found in other fungi like *Arthrobotryx oligospora* which was testified by qRT-PCR (Yang et al., 2018). These results suggest that Mrypt7 may be a positive regulator for

*Monascus* asexual development and the relative regulation genes. While for *Monascus* sexual development process, the *mrypt7*-deletion promoted the expression level of *veA* and *velB*, but didn't activate sexual development as expected (Kim et al., 2002). The following tried to interpret a different sexual development regulation of *M. ruber* M7 may focus on the actual function of *veA* and *velB*, and try to find extra regulators coordinate to cleistothecia formation.

In this study, results indicated that the SMs biosynthesis was also regulated by Mrypt7. Transcriptome analysis showed that Mrypt7 had different impact on the expression of the 9 putative *pks* genes in *M. ruber* M7. Among them, Mps and CIT biosynthesis gene clusters and biosynthesis pathways have been delineated before (He and Cox, 2016; Chen et al., 2017), even both Mps and CIT in  $\Delta mrypt7$  were accumulated in the cell, the expression of Mps and CIT biosynthesis gene clusters showed variant expression level when the *mrypt7* gene was deleted. Researches presented that vesicle localized enzyme were necessary for SMs biosynthesis until they were eventually turned over in vacuoles (Chanda et al., 2009b; Roze et al., 2011), based on the mycelial morphology and



**TABLE 2** | The putative 9 differential expression PKS genes in *M. ruber* M7.

PKS ID	Homologs and related description	Evaluation index	M7-3d vs. M7-7d	$\Delta mrypt7$ -3d vs. $\Delta mrypt7$ -7d	M7-3d vs. $\Delta mrypt7$ -3d	M7-7d vs. $\Delta mrypt7$ -7d
GME1661	Conidial yellow pigment biosynthesis polyketide synthase of <i>Byssoschlamys spectabilis</i> No. 5	log <sub>2</sub> Ratio	0.91	6.48	-0.47	5.10
		Regulation	Up	Up*	Down	Up*
		Probability	0.45	0.99	0.23	0.98
GME2523	Similar to part of lovastatin diketide synthase from <i>Aspergillus terreus</i>	log <sub>2</sub> Ratio	1.61	1.16	-1.39	-1.84
		Regulation	Up*	Up	Down	Down
		Probability	0.81	0.25	0.28	0.58
GME2757	Similar to citrinin polyketide synthase of <i>Monascus purpureus</i>	log <sub>2</sub> Ratio	-0.87	2.31	-0.01	3.17
		Regulation	Down	Up*	Down	Up*
		Probability	0.51	0.94	0.02	0.93
GME4561	Similar to <i>Monascus</i> pigment biosynthesis polyketide synthase of <i>Monascus pilosus</i>	log <sub>2</sub> Ratio	-3.81	0.47	-1.32	2.96
		Regulation	Down*	Up	Down*	Up*
		Probability	0.98	0.87	0.96	0.98
GME6078	A putative polyketide synthase	log <sub>2</sub> Ratio	-0.46	0.78	-1.94	-0.71
		Regulation	Down	Up	Down	Down
		Probability	0.40	0.44	0.76	0.48
GME6749	Similar to putative lovastatin nonaketide synthase of <i>Glarea lozoyensis</i> 74030	log <sub>2</sub> Ratio	-0.18	0.04	-3.33	-3.11
		Regulation	Down	Up	Down*	Down*
		Probability	0.24	0.02	0.85	0.81
GME7032	Similar to lovastatin nonaketide synthase of <i>Fusarium oxysporum</i>	log <sub>2</sub> Ratio	-2.25	1.62	-2.36	1.51
		Regulation	Down	Up	Down	Up
		Probability	0.53	0.44	0.59	0.38
GME7327	A putative polyketide synthase	log <sub>2</sub> Ratio	-0.48	0.64	-0.18	0.94
		Regulation	Down	Up	Down	Up
		Probability	0.09	0.18	0.05	0.21
GME7426	A hybrid PKS-NRPS	log <sub>2</sub> Ratio	0.39	1.66	-0.61	0.66
		Regulation	Up	Up	Down	Up
		Probability	0.05	0.22	0.06	0.11

\*The DEGs reaching the significantly difference levels (log<sub>2</sub>-fold change >1 and probability>0.8).

transcriptome results in this study, it's a reasonable statement that the level of the enzyme and the SMs production were affected by these relative genes expression level which regulated by Ypt7.

sBesides, it's proved that Rab/Ypt protein, SNARE, tethering factors and Sec1/Munc18-family protein worked together to mediate the intracellular destination of a transport vesicle (Baker et al., 2015; Milosevic and Sørensen, 2015; Baker and Hughson, 2016). In this study, four SNARE genes which were important for the transportation on Golgi and endosome (*bet1*, *bos1*, *stx16*, and *stx7*) and three tethering factors genes (golgins, vacuolar protein sorting 22 and transport protein particle complex 10) were differential expressed when *mrypt7* gene was deleted (Table S3). Moreover, syntaxin 16 (Stx16), the important members of SNARE complex, which had been proved to mediate early/recycling endosome to trans-Golgi network and late endosome to trans-Golgi network traffic (Chen et al., 2010), was differential expressed when *mrypt7* gene was deleted (Figure S6), these results suggest that Mrypt7 is functional in both in early and late endosomes. Mrypt7 and the above mentioned

SNARE and tethering factor may work together to finish the fusion process and mediate SMs transportation. The further investigation of the interactions between these proteins could help to develop the detail model of Mrypt7 function in SMs transportation.

It's proved that Ypt family is stable from 7 to 12 Ypts in fungi, except Ypt7, others Ypts (Ypt2, Ypt5, Ypt6, etc.) also affect vegetative growth and conidiogenesis (Wakade et al., 2017; Yang et al., 2017), but had little influence on related genes (Yang et al., 2018). What's more, the Ypt7 disruption had no obvious effect on the expression of the rest of Ypts (Ypt1-Ypt6) in *M. ruber* M7, only Ypt3 was up-regulated in M7-7d vs.  $\Delta mrypt7$ -7d (Table S3). The results further proved that Ypt7 worked more like a global regulator.

Based on the above results, a model of Ypt7 regulation physiological processes was proposed in this study (Figure 4). Briefly, Ypt7, a single-subunit small GTPase, worked as a global regulatory factor, is required for the development, secondary metabolism and vesicle fusion of *Monascus*. First, Ypt7 is a positive regulator for fungal development. The conidiogenesis is suppressed combined with the relative genes (*brlA*, *wetA*,

**TABLE 3** | The differential expression of the Mps biosynthesis gene cluster genes in *M. ruber* M7.

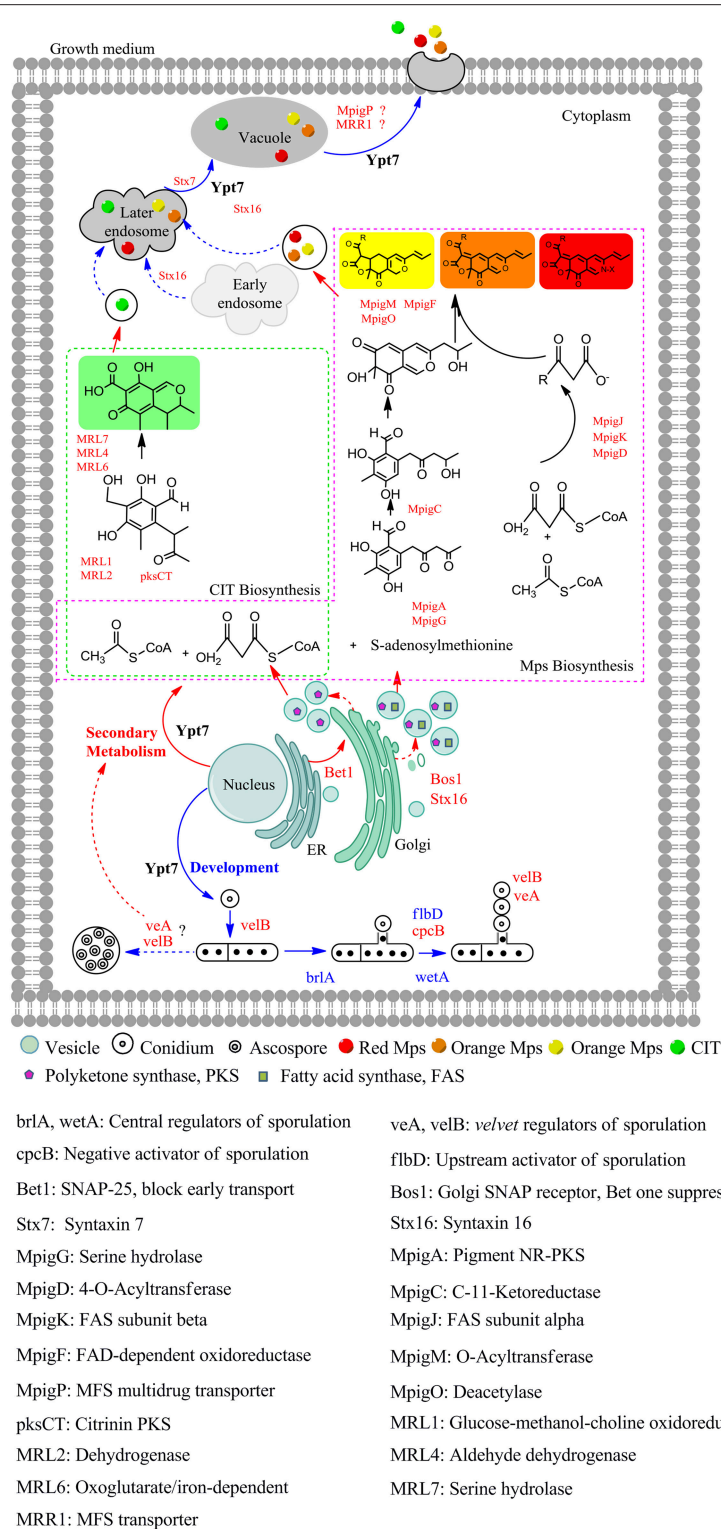
Gene ID	Function description	Up-down-regulation				
		M7-3d vs. M7-7d	$\Delta$ mrypt7-3d vs. $\Delta$ mrypt7-7d	M7-3d vs. $\Delta$ mrypt7-3d	M7-7d vs. $\Delta$ mrypt7-7d	
GME4561	MpigA	NR-PKS	Down*	Up	Down*	Up*
GME4562	MpigB	Transcription factor	Down*	Down	Down	Up*
GME4563	MpigC	C-11-Ketoreductase	Down*	Up	Down*	Up*
GME4564	MpigD	4-O-Acyltransferase	Down*	Down	Down	Up*
GME4565	MpigE	NAD(P)H-dependent oxidoreductase	Down*	Down	Down*	Up*
GME4566	MpigF	FAD-dependent oxidoreductase	Down*	Down	Down*	Up*
GME4567	MpigG	Serine hydrolase	Down*	Up	Down	Up*
GME4568	MpigH	Enoyl reductase	Up	Up*	Down*	Down
GME4569	MpigI	Transcription factor	Up	Up	Up	Up
GME4570	MpigJ	FAS subunit alpha	Down*	Up	Down	Up*
GME4571	MpigK	FAS subunit beta	Down*	Up	Down	Up*
GME4572	MpigL	Ankyrin repeat protein	Down	Up*	Down*	Up*
GME4573	MpigM	O-Acyltransferase	Down*	Up	Down	Up*
GME4574	MpigN	FAD-dependent monooxygenase	Down*	Up	Down*	Up*
GME4575	MpigO	Deacetylase	Down*	Down	Down	Up*
GME4576	MpigP	MFS multidrug transporter	Down*	Up	Down	Up*

\*The DEGs reaching the significantly difference levels ( $\log_2$ -fold change > 1 and probability > 0.8).

**TABLE 4** | The differential expression of the CIT biosynthesis gene cluster genes in *M. ruber* M7.

Gene ID	Function description	Up-down-regulation				
		M7-3d vs. M7-7d	$\Delta$ mrypt7-3d vs. $\Delta$ mrypt7-7d	M7-3d vs. $\Delta$ mrypt7-3d	M7-7d vs. $\Delta$ mrypt7-7d	
GME2750	MRL7	Serine hydrolase	Down*	Up*	Up	Up*
GME2751	MRL6	Oxoglutarate/iron-dependent dioxygenase	Down	Up*	Down	Up*
GME2752	MRL5	Transcription factor	Up*	Up*	Down*	Up
GME2753	MRL4	Aldehyde dehydrogenase	Down*	Up*	Down	Up*
GME2754	MRL3	Aldoketomutase	Down	Up	Up	Up*
GME2755	MRL2	Dehydrogenase	Down*	Up*	Down	Up*
GME2756	MRL1	Glucose-methanol-choline oxidoreductase	Down*	Up*	Down	Up*
GME2757	pksCT	Citrinin PKS	Down	Up*	Down	Up*
GME2758	MRR1	MFS transporter	Up	Up*	Up	Up
GME2759	MRR2	Phosphoglycerate mutase	Down*	Down*	Up	Up
GME2760	MRR3	Hypothetical protein	Up	Up	Up*	Up
GME2761	MRR4	WD repeat protein	Up*	Up	Up	Up
GME2762	MRR5	Carbonic anhydrase	Down*	Down*	Down	Down*
GME2763	MRR6	Hypothetical protein	Down	Down	Up	Up
GME2764	MRR7	Enoyl reductase	Up	Up	Down	Down
GME2765	MRR8	Long-chain fatty acid transporter	Up	Up	Down	Up

\*The DEGs reaching the significantly difference levels ( $\log_2$ -fold change > 1 and probability > 0.8).



**FIGURE 4 |** The proposed model of Ypt7 regulation physiological processes in fungi. The proteins and arrows marked in red indicating that they are up-regulated, the proteins and arrows marked in blue indicating that they are down-regulated, the proteins and arrows marked in black indicated the proved pathways. Dotted lines mean the supposed processes, solid lines mean the experimental processes in current study.

*cpcB*, *flbD*, *veA*, and *velB*) differential expression when Ypt7 was deleted, more remarkable, the sexual development is still suppressed even the sexual active regulators (*veA* and *velB*) were up-regulated which suggested that the sexual development was more rely on the Ypt7 functional completeness (Yang et al., 2018). Besides, *LaeA*, the well-known global regulator, impacts asexual and sexual reproduction but has no noticeable effect on these genes (*bmlA*, *wetA*, *cpcB*, *flbD*, *veA*, and *velB*) (Liu et al., 2016). Second, Ypt7 is a negative regulator for secondary metabolism, the SMs production remarkable rose when Ypt7 was deleted. Ypt7 disruption caused vesicles quantity significantly increased which may increase the vesicle localized SMs enzymes (Roze et al., 2011), and promoted the expression level of SMs biosynthesis gene (Yang et al., 2018). Third, Ypt7 regulates the early transport and later vesicle fusion simultaneously. the early transport between endoplasmic reticulum (ER) and Golgi apparatus was effected by Ypt7 connecting with some SNARE genes, the up-regulate of *Bet1* (blocked early transport) and *Bos1* (*bet* one suppressor) could help to alleviate the lethality associated with disruption of Ypt7 (Newman et al., 1990; Chung et al., 2018). The vesicle fusion and SMs secretion is hampered, but a small quantity of extracellular Mps and CIT can also be detected. Except the known role of Ypt7 in vesicle fusion, two up-regulated syntaxins (*Stx7* and *Stx16*) (Chen et al., 2010) and up-regulated *MpigP* and *MRR1* (multidrug transporters) were supposed to help Mps and CIT secretion.

In a conclusion, this study has indicated the effect and regulation model of *ypt7* gene on vegetative growth, conidiogenesis, vesicle fusion and SMs biosynthesis and transportation in *Monascus*. This is the first comprehensive analysis of the Rab/Ypt family in *Monascus*, the results could enrich the understanding of the function of Rab/Ypt family and make some contribution to uncover the SMs biosynthesis and transportation process in filamentous fungi.

## AUTHOR CONTRIBUTIONS

FC and YZ managed the project. JL and ML performed the transformants construction, secondary metabolites analysis and transcriptome results analysis in this work. JL performed the phenotypic characterization, interpreted the analysis results, and wrote the paper. All authors reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

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**Figure S1** | The schematic diagram of the construction of *mrypt7* deletion (A) and overexpression strains (B).

**Figure S2** | Sequence analysis and characterization of *mrypt7* in *M. ruber* M7. (A) Gene structure analysis by softberry. CDSf, first (starting with start codon) coding segment; CDSi, internal (internal exon) coding segment; CDSl, last (ending with stop codon) coding segment; CDSo, gene contains the ONE coding exon only; PoIA, terminal polyA signal; TSS, transcription start site. (B) Characteristic motifs or residues of Ypt7 in the choosed 285 fungi. phosphate/Mg<sup>2+</sup> binding domain (PM), GTP/GDP binding domains (G) and C-terminal isoprenylation region (C).

**Figure S3** | The mainly pigments of *M. ruber* M7 detected by UPLC. (A) The chromatogram of 4 main yellow pigments at 380 nm which are indicated by 1, 2, 3, and 4; (B) The chromatogram of 2 main orange pigments at 470 nm which are indicated by 5 and 6; (C) The chromatogram of the 2 main red pigments at 520 nm which are indicated by 7 and 8; (D) The chemical structure formula of the 8 pigments.

**Figure S4** | *Monascus* pigments yield analysis of *M. ruber* M7,  $\Delta$ *mrypt7* and M7::PtrpC-*mrypt7*. (A) The yield of intracellular Monasfluore A. (B) The yield of extracellular Monasfluore A. (C) The yield of intracellular Monascline. (D) The yield of extracellular Monascline. (E) The yield of intracellular Monasfluore B. (F) The yield of extracellular Monasfluore B. (G) The yield of intracellular Ankaflavin. (H) The yield of extracellular Ankaflavin. (I) The yield of intracellular Rubropunctatin. (J) The yield of extracellular Rubropunctatin. (K) The yield of intracellular Monascuburin. (L) The yield of extracellular Monascuburin. (M) The yield of intracellular Rubropunctamine. (N) The yield of extracellular Rubropunctamine. (O) The yield of intracellular Monascuburamine. (P) The yield of extracellular Monascuburamine. The error bar represents the standard deviation between the three repeats. Capitals signify *p*-value < 0.01.

**Figure S5** | The validation of transcriptome data by qRT-PCR. (A) The expression level of the ten selected genes in M7-3d VS  $\Delta$ *mrypt7*-3d. (B) The expression level of the ten selected genes in M7-7d VS  $\Delta$ *mrypt7*-7d. The expression level of the genes in M7 was set as 1.

**Figure S6** | The SNARE interactions in vesicular transport.

**Table S1** | Ypt homologous genes in the *M. ruber* M7 genome.

**Table S2** | Primers used for the deletion and overexpression of *mrypt7* gene.

**Table S3** | The proposed genes involved in vesicle transport.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Biopreservative Efficacy of Bacteriocin GP1 of *Lactobacillus rhamnosus* GP1 on Stored Fish Filets

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The bacteriocin based strategy of biopreservation has got wide spread research interests in the recent past for their prospects in reducing usage of chemical preservatives. The bacteriocin GP1 with antibacterial activity and produced by *Lactobacillus rhamnosus* (*L. rhamnosus*) GP1 was tested for its effect on sensory (color, odor, and appearance), chemical (pH, Total Volatile Base-Nitrogen (TVB-N), Total Methyl Amine (TMA), Total Free Fatty Acid) and bacteriological (total bacterial count, count of *Staphylococcus* sp., *Aeromonas* sp., total coliform, *Lactobacillus* sp., *Pseudomonas* sp., and *Vibrio* sp.) quality attributes of fish filets stored at 4 and 0°C. The sensory attributes of the fish filets treated with the bacteriocin and control from 7 to 28 days of storage in both the storage temperatures varied significantly. The pH of the raw fish increased from the initial 6.8 to 7.91 and 7.43 for the control and bacteriocin GP1, respectively, at the end of storage period (28 days) when stored at 4°C. However, the pH showed a decreasing trend with the increase in period of storage for the samples stored at 0°C. The TVB-N content of the bacteriocin treated samples stored at 4°C remained within the limit of acceptability (35 mg/100g) at the 21st day. The TMA level also remained within the acceptable limit of 10–15 mg/100g at the 21st day in the case of bacteriocin-treated samples. The application of bacteriocin GP1 in the stored fish was effective in controlling the growth of coliforms, *Aeromonas* sp., *Lactobacillus* sp., and *Vibrio* sp. in the treated fish samples. The study concluded the prospects of bacteriocin GP1 as a biopreservative in storage of fish and fish products.

**Keywords:** *Lactobacillus rhamnosus* GP1, bacteriocin GP1, Nisin B440, sodium benzoate, biopreservative, fish filets

## INTRODUCTION

The seafood industry is constantly searching new technologies for storage of fresh fish as they are highly perishable due to their biological composition. Chemical preservatives and other conventional preservation strategies fail to deliver the requisite health benefits and cause serious disorder thus necessitates seeking alternatives. The major cause for the spoilage of fresh and preserved fish is the microbial growth and metabolic activity (1). Recent research on to the same centers around the biopreservative strategy which consists of inoculating food product by selected bacteria and its antibacterial products which deter the growth of spoilage causing micro-organisms, without altering the product quality (2).

Lactic acid bacteria (LAB) have huge prospects for use in biopreservation. The GRAS status together with the conviction of being safe to consume permits their usage in foods without additional regulatory approval. Bacteriocins of LAB with potent antibacterial activity against food spoilage and pathogenic bacteria have been used as a natural food ingredient, essentially as a biopreservative. The bacteriocins have been consumed for millennia by mankind as products from LAB which relates the fact that the only commercialized food grade bacteriocins available till date are purified from them. Further, being peptides, bacteriocins are assumed to be easily degraded by the proteases in the gastrointestinal tract and hence are considered as good biopreservatives. Most of the studied bacteriocins possess good thermo stability thus able to remain stable during thermal processing cycle of foods, while the others can act at low pH and temperature thus finds application in acid foods and cold-stored products (3). Many bacteriocins have been isolated for use as natural food biopreservative (4, 5). Nisin produced by *Lactococcus lactis* sub sp. *lactis*, is a bacteriocin approved for application in the food industry for its activity against Gram positive foodborne pathogenic microorganisms (6). Previous studies have shown the effective application of nisin as a biopreservative in processed fish (7–9).

The inhibitory potential of the bacteriocin GP1 produced by the LAB, *Lactobacillus rhamnosus* (*L. rhamnosus*) GP1 has been demonstrated against a number of spoilage-producing and pathogenic bacteria (10). This study was aimed to find out the effect of application of the bacteriocin GP1 on microbiological, chemical, and sensory quality of high value fish filets stored at 0 and 4°C in comparison with the chemical preservative sodium benzoate and the bacteriocin nisin B440.

## MATERIALS AND METHODS

### Growth and Culture of Bacteria

The bacteriocin producing LAB strain (*L. rhamnosus*) GP1 (10) was grown in MRS medium (11) and incubated at 30°C. *L. brevis* BF1 was used as the indicator strain to assay the bacteriocin. Both the strains were stored in MRS agar (Hi-Media) slants and propagated as and when required.

### Bacteriocin Preparation and Determination of Activity

Five milliliters of working culture of *L. rhamnosus* GP1 was transferred to 1,000 ml of modified MRS broth (supplemented with 0.1% w/v of yeast extract, 0.1% v/v of tween 80, and 2.0% w/v of glucose) to maximize the production of bacteriocin. The culture broth was centrifuged after growth (72 h at 30°C; pH 3.75) at 10,000 rpm for 30 min and the cell free culture supernatant was collected. The supernatant fluid was adjusted to pH 7.0 with 0.1 N NaOH and was filter sterilized (0.45 µm pore size) under vacuum. The bacteriocin GP1 was partially purified by pH-adsorption (12) and activity determined. For detecting the bacteriocin activity, 10 µl of the cell free filtrate was placed on an agar plate containing an overlay of indicator cells. The inhibitory activity against the indicator organisms was observed after incubating the agar plate for 24 h. Antimicrobial activity

was expressed as arbitrary units (AU). Each arbitrary unit is the reciprocal of highest dilution showing a clear zone of growth inhibition (13).

### Fish Preparation and Sampling

The fish used in the study belonged to Grouper (Reef Cod) family and was obtained from Vizhinjam Fish Landing Center, Thiruvananthapuram, Kerala, India. The fish weighing 11.8 kg was immediately brought to the laboratory in insulated container and washed in potable water. The fish as a whole was dipped in chlorine water, washed with sterile water, beheaded and eviscerated. Then it was allowed to bleed for 15 min and washed again in sterile water and skin removed, deboned, and fileted (10 g each) using a sterile sharp knife. The filets were surface sterilized by exposure to UV light at 265 nm (Arklite, India) for 15 min and sterility determined by microbiological examination. Four different preservative solutions were prepared with 800 AU/ml nisin (produced by *Lactococcus lactis* MTCCB440), 1,200 AU/ml bacteriocin GP1, 0.1% of sodium benzoate and autoclaved distilled water which served as the control. The prepared preservative solutions (2 ml each) were sprayed evenly over the fish samples (10 g) with a sterile hand-held sprayer and kept intact for 20 min. The treated fish pieces were enfolded in sterile aluminum foil, placed in separate sterile boxes, and stored at temperatures *viz.*, 4 and 0°C. At 0 (within 20 min of treatment), 7, 14, 21, and 28 days of refrigeration, samples from each treatment were taken out subjected to different analyses—microbiological, physicochemical, and sensory. All the analyses were done in triplicate.

### Analysis of Bacterial Count

For bacteriological analysis, 1 g slice of the stored filet was made using sterile blade and homogenized for 2 min using sterile mortar and pestle. The samples were serially diluted and plated in triplicate on nutrient agar and selective agar plates such as Eosin Methylene Blue (Hi-Media), Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (Hi Media), de Mann Rogosa Sharpe (MRS; Hi Media) agar, and Pseudomonas Selective Agar (Hi Media) at 30°C for 48 h.

### Sensory Evaluation

The odor and appearance of fish samples were analyzed for their sensory characteristics using a 9-point hedonic scale (14); 1 being dislike extremely and 9 like extremely (15) using five trained panelists.

### pH Measurements

The samples stored at different temperatures were subjected to determination of change in pH. The samples (1 g each) was homogenized using 9 ml distilled water and pH determined using a Cyberscan model 500 pH meter (Euteon Instruments, Singapore). The results were expressed as the mean of the determinations.



## Total Volatile Basic Nitrogen (TVB-N) and Total Methyl Amine (TMA) Measurements

Conway’s method was employed for determination of TVB-N and TMA of the samples. For analyses, the 2 g of sample from each experimental set and homogenized with 10 ml of 4% trichloroacetic acid which was filtered through a Whatman No.1 filter paper. The filtrate was used for analyses of TVB-N and TMA. The experiments were carried out following the procedure of Conway (16).

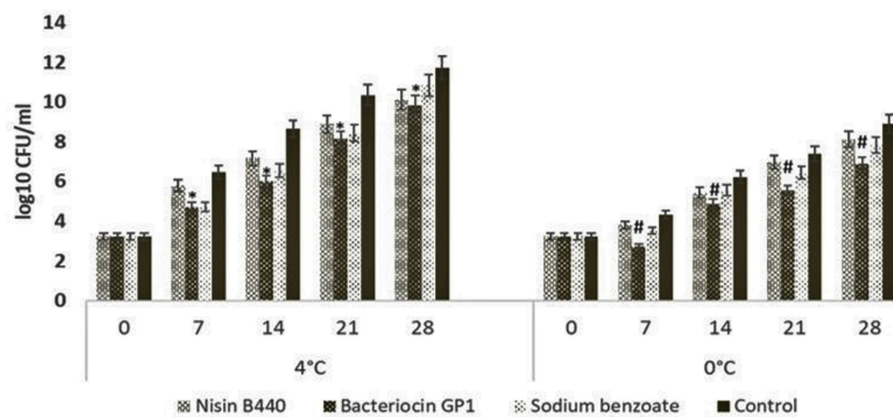
## Statistical Analysis

The experiments were done in triplicate. Data were analyzed using statistical method ANOVA on 5% significance level in Excel 2010.

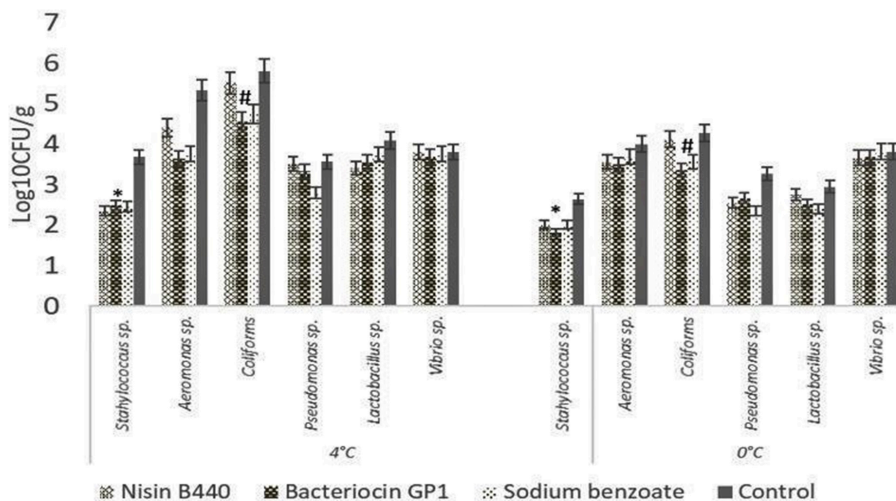
## RESULTS

### Bacteriocin Preparation

The LAB strain (*L. rhamnosus*) GP1 (10) was used to extract the bacteriocin GP1 to study its application as biopreservative in chill-stored high value marine fish. The growth of the strain in modified MRS broth showed a drastic increase between 24 and 60 h after inoculation (log phase). A gradual stationary and a steady decline phase were obtained subsequent to this slope. The maximum production of bacteriocin GP1 was noted in the stationary phase, at 72 h. The partially purified bacteriocin GP1 was collected following centrifugation of the culture and pH-adsorption of the cell free culture supernatant.



**FIGURE 1 |** Change in total viable count of the fish samples stored at 4 and 0°C. The bar graphs show mean ± SD. \*indicates a significant difference between bacteriocin GP1 treated samples and control ( $P = 0.02$ ) stored at 4°C, #indicates a significant difference between bacteriocin GP1 treated samples and control ( $P = 0.019$ ) stored at 0°C.



**FIGURE 2 |** Change in specific bacterial count of stored fish filets on the 7th day of storage. The bar graphs show mean ± SD. \*indicates a significant difference in *Staphylococcus* count between different treatments ( $P = 0.045$ ) stored at 4 and 0°C, #indicates a significant difference in Coliform count between different treatments ( $P = 0.008$ ) stored at 4 and 0°C.

## Microbiological Analyses

The total bacterial count of the fish sample stored at 4 and 0°C are presented in **Figure 1**. The microbial load increased with increase in the storage period at 4°C irrespective of the treatments employed. The initial mean bacterial load was  $3.24 \pm 0.11$  CFU/g in all the experimental groups. Compared with the control which started deteriorating after 7 days of storage, the growth of spoilage bacteria was significantly inhibited in treated samples; the treatment with bacteriocin GP1 and sodium benzoate managed to extend the fish shelf life up to the 14th day of storage.

The results of specific bacterial counts, obtained from samples at the 7th day of storage, are shown in **Figure 2**. The bacteriocin GP1, nisin B440, and the chemical preservative sodium benzoate significantly inhibited the psychrophiles compared to the control. The psychrophilic bacterial count increased with increase in storage period up to 28 days (data not shown). The load of *Staphylococcus* sp., *Aeromonas* sp., coliforms, *Lactobacillus* sp., *Pseudomonas* sp., and *Vibrio* sp. followed an increasing trend initially up to the 14th day, which became stable till 28th day. While a reduction in the spoilage bacterial count was noted for all the treated samples compared to the control, bacteriocin GP1 was effective in controlling the growth of coliforms, *Aeromonas* sp., *Lactobacillus* sp., and *Vibrio* sp.

## Sensory Evaluation

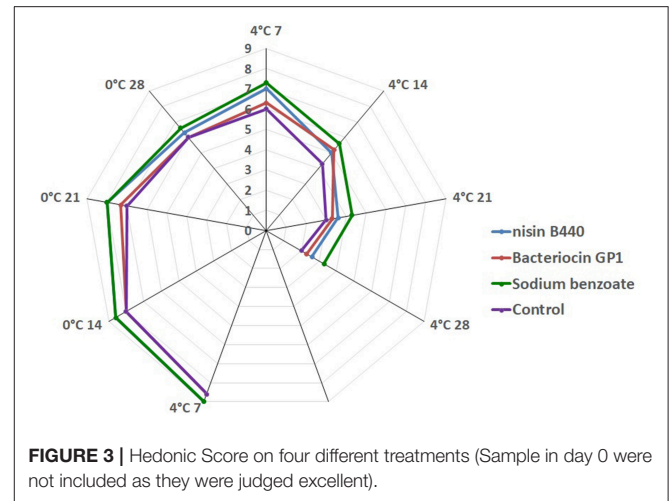
The sensory scores decline with storage (**Figure 3**). Though, the level of acceptability of the fish sample decreases as the storage time increases in both the treated and untreated fish samples, significant difference ( $P < 0.05$ ) was noted in the sensory attributes in case of the treated fish samples compared to the control from 7 to 28 days of storage. The fish sample treated with nisin B440, bacteriocin GP1, and sodium benzoate had  $5.0 \pm 0.0$ ,  $5.2 \pm 0.4$ , and  $5.6 \pm 0.4$  score, respectively at day 14 of storage at 4°C while the control had  $4.3 \pm 0.4$  score at the same time of storage. The overall scores were beyond the acceptable limit (4.0) at the end of the storage period (28 days) at 4°C.

## Change in pH

The pH of the fish samples showed an increasing trend with increase in storage period. **Table 1** summarizes the average values analyzed on each sampling day. For the fish samples stored at 4°C, the pH values showed an increased trend in both the control and experimental sets. The range in pH was from  $6.8 \pm 0.01$  to  $7.91 \pm 0.01$  as determined from the initial day to the end of storage period (28th day) in case of control as against the treated samples which maintained the pH range of  $<7.0$  till the 21st day of storage. Significant differences ( $P < 0.05$ ) were noted in the pH of the fish samples treated with the bacteriocin and control from 7 to 28 days of storage in both the storage temperatures.

## Changes in TVB-N

The TVB-N values of the treated samples and control are shown in **Figure 4**. The average TVB-N content in the fish samples was  $4.67 \pm 4.0$  and  $55.33 \pm 4.0$  mg of nitrogen / 100 g of fish flesh, respectively, at 0 and 28 days stored in 4°C. By day 21, the TVB-N value of control had increased from 21 to 37 mg/100 g of



**FIGURE 3** | Hedonic Score on four different treatments (Sample in day 0 were not included as they were judged excellent).

fish. For the bacteriocin treated samples, although it increased with storage; from day 7, it showed a reduced level when compared to the control. For, the samples stored for 21 days, the TVB-N content showed a significant difference ( $P < 0.05$ ) which compared with the control, the values were  $32.67 \pm 4.0$ ,  $30.33 \pm 4.0$ , and  $28.0 \pm 7.0$  mg of Nitrogen/100 g for nisin B440, Bacteriocin GP1 and sodium benzoate treated samples, respectively, as against  $37.0 \pm 4.0$  mg of Nitrogen/100 g observed for the untreated (control) samples. The pattern of TVB-N production in 0°C was consistent with that observed for 4°C in all experiments with significant difference noted ( $P < 0.05$ ) for treated samples vs. control (**Figure 4**), though remained within the limit of acceptability (30–35 mg/100 g) even at the end of storage period.

## Changes in TMA

The measurements made in terms of TMA are presented in **Figure 5**. The TMA content also followed an analogous trend to that of TVB-N when stored at 4°C where a significant increase was observed ( $P < 0.05$ ) with storage period. Initial TMA content was 0 for both the untreated and treated samples. The values in the 28th day were  $20.33 \pm 4.0$ ,  $20.67 \pm 4.0$ , and  $19.5 \pm 0.0$  for Nisin B440, Bacteriocin GP1 and sodium benzoate treated samples, respectively, as against  $25.67 \pm 4.0$  observed in case on the untreated (control) sample. It could also be observed that the TMA values for the samples stored at 0°C were lower than the samples stored at 4°C though significant differences amongst the treatments were noticed as is evidenced statistically. However, the values remained within the range of acceptability (10–15 mg/100 g) even after 28 days of storage at 0°C in both the treated samples and the control.

## DISCUSSION

Biopreservation approaches for food items are gaining importance and interest among the industry and the consumers. The biopreservative efficiency of several bacteriocins had been proved earlier (17–19) in different food systems viz., fish, meat,

**TABLE 1** | pH changes of the fish filets treated with bacteriocin preparations stored at 4 and 0°C for 28 days; the values are shown as show mean ± SD.

Treatments	4°C					0°C*				
	0	7	14	21	28	0	7	14	21	28
Nisin B440	6.8 ± 0.01	6.84 ± 0.01	6.90 ± 0.00	6.99 ± 0.02	7.50 ± 0.01	6.82 ± 0.01	6.76 ± 0.01	6.75 ± 0.01	6.69 ± 0.01	6.62 ± 0.01
Bacteriocin GP1	6.8 ± 0.01	6.82 ± 0.01	6.85 ± 0.01	6.94 ± 0.01	7.43 ± 0.02	6.8 ± 0.01	6.75 ± 0.01	6.73 ± 0.00	6.67 ± 0.01	6.62 ± 0.02
Sodium benzoate	6.8 ± 0.01	6.84 ± 0.01	6.87 ± 0.01	6.98 ± 0.01	7.41 ± 0.01	6.81 ± 0.01	6.76 ± 0.01	6.72 ± 0.01	6.66 ± 0.01	6.60 ± 0.01
Control	6.8 ± 0.01	6.87 ± 0.02	7.01 ± 0.01	7.25 ± 0.01	7.91 ± 0.01	6.82 ± 0.01	6.79 ± 0.02	6.77 ± 0.01	6.70 ± 0.01	6.65 ± 0.01

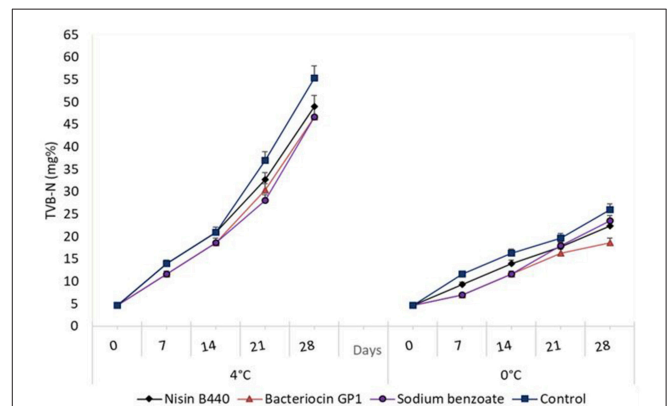
\*Indicates a significant difference in pH between bacteriocin GP1 treated samples and control ( $P = 0.001$ ) stored at 0°C.

and vegetable products. The effectiveness of bacteriocin based biopreservatives had been extensively tested in meat products (19, 20) and in vegetables (21). However, scanty information is known regarding preservation of seafood using bacteriocins. The present study targeted on investigating the preservative efficacy of the studied bacteriocin GP1 produced by (*L. rhamnosus*) GP1 in a high value fish sample stored under different temperatures (4° and 0°C).

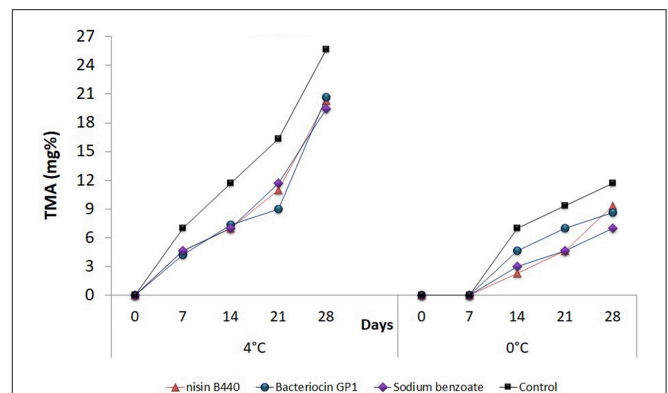
There was an increase in total bacterial count during storage at 4°C irrespective of the treatments employed. The results of increased viable count above  $8 \log_{10}$  CFU/g in all samples after 14 days of storage period were similar to earlier findings (22). The bacterial count could have increased due to the ambient congenial growth conditions at the higher temperatures of storage (23). To the observations of Randazzo et al. (18), the survival and persistence of natural contaminant organisms in the fish is the result of high final load detected throughout the period of storage. Up on comparing the bacterial load among the different treatments, it was evidenced that the bacteriocin treated samples slowed down the bacterial proliferation when compared with the untreated samples, the efficacy of bacteriocin GP1 in controlling the spoilage bacteria is in par with the biopreservative nisin and chemical preservative sodium benzoate. Since the load of bacteria in GP1 treated samples ( $5.99 \pm 0.04 \log_{10}$  CFU/g) remained within the acceptability limit at the 14th day, it could be concluded that this bacteriocin could extent the shelf-life of the fish. The potential of the bacteriocins to control the spoilage bacteria in fish filets are in consistence with the observations made with Pediocin 31-1 (19) in pork meat.

The proliferation of the psychrophilic bacteria (24) in the fish filets occurred with increase in storage period at the higher temperature of 4°C. Liu et al. (25) isolated psychrophiles from tray-packed Tilapia filets stored at 0°C. Similar to this observation, the cod fish flesh stored at 0°C harbored a definite load of the psychrophiles, which followed a stable trend throughout the period of storage in the case of the untreated sample. However, the observations with the bacteriocin treated samples were encouraging in that it could limit the growth of at least a few strains of bacteria.

The sensory characteristics of fish are clearly noticeable to the consumer and are extremely important for consumer satisfaction (26). The observations made for odor and appearance were



**FIGURE 4** | Changes in the total volatile base nitrogen (TVB-N) content (mg%) in fish filets stored at 4 and 0°C; values represent means ± SE, Significant difference is defined at  $P < 0.05$ .



**FIGURE 5** | Changes in the total methyl amine (TMA) content (mg%) in fish filets stored at 4 and 0°C; values represent means ± SE, Significant difference is defined at  $P < 0.05$ .

considered for determining the sensory attributes of the stored fish sample. The fish samples at the initial day i.e., just before storage had the best sensory attributes and scored 9.0. However, the acceptability scores for odor and appearance of the fish stored

at 4 and 0°C decreased with the progression of storage. The fish scored “liked slightly” during the first 7 days of storage and scored “dislike slightly” between 7 and 14 days of storage for control group samples (Figure 3). The fish samples reached the acceptability quality limit of 4.0 as per the protocol of Renitta et al. (27) at the 14th day for the control samples and after 15 days for the bacteriocin treated samples. The fish sample treated with bacteriocin GP1 had  $5.2 \pm 0.2$  score at day 14 at 4°C while the control had  $4.3 \pm 0.4$  score at the same time of storage. There were significant differences in the sensory attributes for the fish samples treated with the bacteriocins and control at the 21st and 28th day of storage, though the samples have become unacceptable.

The pH changes in the fish flesh is determinant of the decomposition of nitrogenous compounds during storage thus producing alkaline compounds. This high post-mortem pH (28, 29) indicates the bacterial growth, loss of quality and possible spoilage. The pH of the raw fish used in the study was in the range of 6.8 in the beginning, which changed drastically upon storage at 4°C. Contrary to observation made at 4°C, the pH showed a decreasing trend with the increase in period of storage for the samples stored at 0°C.

Total volatile bases nitrogen (TVB-N) measurements quantify the contents of trimethylamine (TMA), dimethylamine (DMA), ammonia, and other volatile basic nitrogenous compounds associated with seafood spoilage (30, 31). A significant increase of TVB-N values ( $10.48 \pm 0.07$  mg/100 g) was recorded in the stored fish ( $P < 0.05$ ). The TVB-N content of the untreated (control) fish sample stored at 4°C exceeded the maximum level for acceptability for marine fish i.e., 35 mg/100 g (32), after 21 days. This value agrees with TVB-N levels of sea bass (33) and Malawi Tilapia (34) stored in ice. In the case of the bacteriocin treated samples, it remained within the limit of acceptability at the 21st day of storage. The observations in tune with this had been made earlier in Pediocin 31-1 in which the 80 AU/ml of this bacteriocin maintained TVB-N within the acceptability limit to 15 days as against the control (19).

The content of TMA was below detectable level (0) in the case of cod fish when determined initially. Earlier observation (35) showed TMA content to range from 0.8 to 4 mg/100 g in fresh marine fishes. TMA values increased throughout the time of storage in the present study; the level of 10–15 mg/100 g being the maximum limit of acceptability (36) to indicate fish freshness.

This level was attained in the untreated (control) fish sample at the 14th day, whereas in the bacteriocin treated sample remained within the limit of acceptability even at the 21st day of storage and thereafter became unacceptable. Thus, it was noted that the bacteriocin GP1 was most effective in restricting the production TVB-N and TMA, thereby increasing the shelf-life of the fish at 4°C.

The reduction in the load of the bacteria and all other parameters observed with the bacteriocin treated samples were analogous with the experiments conducted with the chemical preservative sodium benzoate as is evidenced from the results of this study. The preservation of the fish at the ambient ( $25 \pm 2^\circ\text{C}$ ) or lower temperatures (4°C) requires the use of chemical preservatives viz., benzoates, nitrites, sulphites, and sorbates (37) or tedious processing (38) steps to enable the shelf-life extension. But as the chemical preservatives are associated with undesirable side effects (39), a replacement is required which does not influence the organoleptic attributes of the product. The findings made in this study could be considered noteworthy as there is a likelihood that bacteriocin GP1 could be further purified and used as preservative for storing high value marine fish.

## DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Immunoreactivity of Gluten-Sensitized Sera Toward Wheat, Rice, Corn, and Amaranth Flour Proteins Treated With Microbial Transglutaminase

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The aim of this study was to analyze the effects of microbial transglutaminase (mTG) on the immunoreactivity of wheat and gluten-free cereals flours to the sera of patients with celiac disease (CD) and non-celiac gluten sensitivity (NCGS). Both doughs and sourdoughs, the latter prepared by a two-step fermentation with *Lactobacillus sanfranciscensis* and *Candida milleri*, were studied. In order to evaluate the IgG-binding capacity toward the proteins of the studied flours, total protein as well as protein fractions enriched in albumins/globulins, prolamins and glutelins, were analyzed by SDS-PAGE and enzyme-linked immunosorbent assay (ELISA). Results showed that while mTG modified both gluten and gluten-free flour by increasing the amount of cross-linked proteins, it did not affect the serum's immune-recognition. In fact, no significant differences were observed in the immunoreactivity of sera from CD and NCGS patients toward wheat and gluten-free protein extracts after enzyme treatment, nor did this biotechnological treatment affect the immunoreactivity of control samples or the sera of healthy patients. These results suggest that mTG may be used as a tool to create innovative gluten and gluten-free products with improved structural properties, without increasing the immune-reactivity toward proteins present either in doughs or in sourdoughs.

**Keywords:** transglutaminase, celiac disease, cereal food, gluten-free, sourdough

## INTRODUCTION

Celiac disease (CD) and non-celiac gluten sensitivity (NCGS) can be regarded as immune-mediated systemic disorders elicited by gluten and related prolamins, among which deamidated gliadins are the most immunogenic proteins (Volta et al., 2013). Epidemiological analysis on populations living in western countries reported that 1% of the European population suffers from CD (Kurien et al., 2016) while the prevalence of NCGS is still far from being established, ranging from 1% (primary

care) to 6% (tertiary care) (Volta et al., 2014). Immune-mediated mechanisms are triggered by gluten and evoke intestinal mucosal damage resulting in villous atrophy in patients with CD. This histopathological change is associated with markedly reduced transmucosal absorption ending with nutrient deficiency. Currently, the only therapeutic option for patients with CD is a lifelong strict adherence to a gluten-free (GF) diet (Dowd et al., 2014). Less information is available for NCGS, although some authors have suggested an identical dietary approach for NCGS patients. On the other hand, the quality of baked products, in terms of viscosity, elasticity and cohesion, is strictly dependent on gluten. Indeed, its capacity to create protein aggregates and thus to confer structure and texture to the dough, is a relevant feature for the quality of baked products (Delcour et al., 2012). Producing GF baked goods with preserved palatability and a good nutritional profile is the modern challenge for food technology.

When added to dough, cross-linking enzymes such as transglutaminases (TGs) can create protein networks contributing to the structure of the dough; for this reason, TGs are considered a promising tool for the food industry. TGs are a widely distributed family of enzymes (E.C. 2.3.2.13) (Del Duca and Serafini-Fracassini, 2005), belonging to the class of transferases. Even if TGs from plant and microorganisms lack sequence identity in respect to mammalian ones, they share similar reactions and functions. In fact, either plant or mTGs have conserved cysteine, histidine, and aspartate residues that form the catalytic triad in structurally characterized TGs (Makarova et al., 1999; Serafini-Fracassini et al., 2009).

TGs catalyze three post-translational protein modifications, namely (i) transamidation, (ii) deamidation of endoglutamine residues, and/or (iii) cross-linking between glutamine and lysine residues (intra- or inter-chain), giving rise to protein aggregates (Lorand and Graham, 2003). TGs are also involved in various biological processes and clinical applications (Brunner et al., 2002; Facchiano et al., 2006; Del Duca et al., 2014); further, they are targets in therapeutic developments (Martins et al., 2014; Strop, 2014).

Since the discovery of the first microbial TG (mTG) in 1989 (Ando et al., 1989), many efforts have been made in the selection of mTG-producing strains and in the optimization of the mTG production process (Motoki and Seguro, 1998) with the aim of obtaining low-cost enzymes suitable for food industry applications.

Nowadays, mTG is an important tool in the food industry (Martins et al., 2014) as well as for research and biotechnological applications (Camolezi Gaspar and Pedroso de Góes-Favoni, 2015). Improving firmness, viscosity, elasticity, and water-binding capacity of food products in order to increase organoleptic features, is of great interest in the food industry. Thanks to their protein cross-linking reactions, mTGs have been shown to be a suitable tool for food treatment (Kieliszek and Misiewicz, 2014). Recently, mTG treatment was also shown to improve the structure of GF dough by inducing protein aggregation via protein cross-linking reactions (Scarnato et al., 2016). Moreover, we reported the effects of the combination of mTG and sourdough on the rheological properties, aroma, and

shelf-life of wheat bread (Scarnato et al., 2017) and the same food technology has been applied on GF dough, which showed an improved structure (Scarnato et al., 2016). Considering that mTG is not only able to cross-link proteins, but also to catalyze the deamidation of glutamine residue, the latter modification of the mTG-treated products cannot be excluded a priori (Skovbjerg et al., 2004; Gerrard and Sutton, 2005; Heil et al., 2016).

The aim of this research was to evaluate the capacity of mTG to modify wheat and GF proteins by catalyzing protein cross-links and to identify potential changes in the IgG immunoreactivity of those proteins after biotechnological treatment.

## MATERIALS AND METHODS

### Flours and Doughs Preparation

Barilla S.p.A. (Parma, Italy) provided straight-grade wheat flour; corn, rice and amaranth flours were purchased from local markets. Flour doughs were prepared by mixing flour and water (control dough); sourdough were prepared by adding *L. sanfranciscensis* and *C. milleri* strain to the dough, as described previously (Scarnato et al., 2016; Scarnato, 2017).

### mTG Treatment of Flour Dough Proteins

Ajinomoto kindly provided the mTG, Activa WM (acTG) from to *Streptomyces mobaraensis*, (specific activity: 81–135 U/g, Ajinomoto Foods Europe S.A.S., France).

Treatment of doughs with mTG were carried out by mixing 1U of enzyme/100 mg flour at 40°C for 90 min with constant stirring. Then, treated doughs were stored at –20°C in order to stop mTG-activity or immediately processed for protein extraction.

### Sera Used for Immunological Analysis of Flour Proteins

A collection of sera from blood samples taken for diagnostic purposes was identified in the serum bank of the Clinical Immunology Laboratory (Department of Medical and Surgical Sciences, St. Orsola-Malpighi Hospital of the University of Bologna). The samples included for this study were selected from sera labeled as “CD patients” ( $n = 14$ ), “NCGS patients” ( $n = 17$ ), and “healthy control blood donors” (HCBD) ( $n = 6$ ). IgA anti-TG2 antibodies (TGA), IgG anti-deamidated gliadin peptides antibodies (DGP) and anti-endomysium antibodies (EmA) showed positivity along with the presence of duodenal villous atrophy in patients diagnosed with CD following a gluten-containing diet. NCGS patients were diagnosed following the Salerno Experts Criteria (Catassi et al., 2015). In detail, patients with NCGS are identified as subjects with gluten-related symptoms that rapidly improved after gluten withdrawal and in which CD and wheat allergy have been ruled out. The symptom improvement was considered indicative of NCGS if the score obtained from the modified version of the gastrointestinal symptom rating scale (GSRS), including extra-intestinal symptoms, decreased by at least 30% from baseline after a gluten-free diet (GFD). In addition, positivity for anti-native gliadin antibodies (AGA) of the IgA and/or IgG class, although not specific, is regarded as another tool supporting the NCGS diagnosis. Correct labeling

of the selected sera was checked by retesting each sample for TGA, EmA, DGP, AGA, and specific IgE for foods and aeroallergens.

Since we used sera from anonymous blood samples taken for diagnostic purposes, an approval from the St. Orsola-Malpighi Ethics committee was deemed unnecessary. HCBd gave written informed consent prior to blood sampling.

The collection of 36 human sera is listed in **Table 1**. Pooled sera, containing individual serum from different groups (CD, NCGS, and HCBd), were prepared by adding the same amount of serum from each of the three types.

## Serological Tests Performed With Human Sera

Immunoglobulin A tissue transglutaminase antibodies (tTGA) were measured using a commercially available ELISA kit (EuTG IgA, Eurospital, Trieste, Italy), using recombinant human tissue TG as antigen. A cut-off value of 16 arbitrary units (AU), provided by the manufacturer, was adopted.

Immunoglobulin G deamidated gliadin peptide antibodies (DGP) were assessed by ELISA using commercially available kits (a-glia PEP, Eurospital, Trieste, Italy) and an entirely synthetic peptide constructed in a conformational intact manner and then selectively deamidated. According to the manufacturer's instructions, the cut-off value was set at 16 AU.

Immunoglobulin A endomysial antibodies (EmA) were investigated by indirect immunofluorescence using human umbilical cord cryostat sections (4 mm), cut in our laboratory, as substrate. Sera were tested at the initial dilution of 1:5 and, when positive, titrated to the end point.

Immunoglobulin A and Immunoglobulin G anti-gliadin antibodies (AGA) were determined by ELISA using commercially available kits (a-gliatest SIgA and SIgG, Eurospital, Trieste, Italy) and purified a-gliadin as antigen. The cut-off levels, as suggested by the manufacturer, were fixed at 50 and 15 AU for IgG and IgA AGA, respectively.

## Protein Extraction and Dialysis

Proteins from dough, treated with mTG, were extracted with different buffers (1 g of flour/10 mL of buffer) in order to obtain total protein extracts (TE) and fractions enriched in albumins and globulins (F1), prolamins (F2) and glutelins (F3). All steps were carried out at 4°C. To prepare total protein extract, the dough was suspended in 100 mmol/L Tris HCl pH 8.5 containing 20% glycerol and 1.7% β-mercaptoethanol. The mixture was subjected to ultra-sonication for 30 s on ice, and then incubated overnight with constant stirring. The supernatant was collected after centrifugation at 5,000 g for 10 min and then dialyzed against 0.1 mol/L acetic acid for 24 h using 6–8 kDa cut-off dialysis membranes. The protein-enriched fractions were obtained using the same above-described procedure but using different extraction buffers as described by Rallabhandi et al. (2015), with minor modifications. First, the dough was extracted twice with 0.5 mol/L NaCl pH 7.5 for 1 h. The two supernatants, containing the F1-enriched fraction, were pooled before dialyzing against distilled water. The residual extraction pellet was washed with water

for 10 min followed by centrifugation. Then, the pellet was resuspended twice in 70% ethanol for 1 h. The extracts containing prolamins (F2) were pooled and the solvent was removed under vacuum. Finally, the glutelin fraction (F3) was extracted by resuspending the residual pellet twice in 50% isopropanol, 1% acetic acid, 0.5% β-mercaptoethanol, followed by dialysis against 0.1 mol/L acetic acid. All the protein extracts were stored at –20°C until further use. The protein content was estimated using Bradford's method with BSA as standards (Bradford, 1976).

**TABLE 1** | Clinical diagnosis and serum antibodies of patients.

ID	Diagnosis	EmA IgA	tTG IgA	DGP-IgG	AGA IgA	AGA IgG
1	CD	pos+++	112.8	nd	nd	nd
2	CD	POS+++	31.6	69.1	7.1	29.3
3	CD	POS+++	132.2	144.3	30.8	151.3
4	CD	POS+--	22.1	68.5	nd	nd
5	CD	nd	139.6	55.6	nd	nd
6	CD	POS+++	116.9	39.6	3.9	42.7
7	CD	nd	135.9	115.9	nd	nd
8	CD	nd	79.4	113.2	nd	nd
9	CD	POS+++	152.8	101.6	28.5	138.9
10	CD	nd	132.8	61.3	nd	nd
11	CD	POS+++	151.9	106.4	19.1	104.2
12	CD	POS+++	150.9	96.4	49.1	139.6
13	CD	POS+--	105.7	nd	nd	nd
14	CD	nd	121.7	17.2	nd	nd
15	NCGS	NEG	4.8	5.1	4.6	40.1
16	NCGS	NEG	4.0	9.5	8.8	114.9
17	NCGS	NEG	5.4	36.0	12.7	65.1
18	NCGS	NEG	5.6	19.7	8.5	34.1
19	NCGS	NEG	4.5	7.9	6.0	47.8
20	NCGS	NEG	5.3	3.4	8.8	44.4
21	NCGS	NEG	5.0	26.0	5.9	85.8
22	NCGS	NEG	5.2	5.3	8.0	41.6
23	NCGS	NEG	5.8	7.6	4.6	51.7
24	NCGS	NEG	4.5	4.5	7.3	50.7
25	NCGS	NEG	4.5	7.0	7.0	87.0
26	NCGS	NEG	5.1	7.6	6.3	40.7
27	NCGS	NEG	2.4	4.1	5.1	54.1
28	NCGS	NEG	5.7	10.0	8.3	43.0
29	NCGS	NEG	5.9	22.8	8.0	119.0
30	NCGS	NEG	4.2	3.3	6.6	54.1
31	NCGS	NEG	5.2	3.4	7.8	88.5
32	HCBd	NEG	1.0	nd	nd	nd
33	HCBd	NEG	0.5	nd	nd	nd
34	HCBd	NEG	1.5	nd	nd	nd
35	HCBd	NEG	1.0	nd	nd	nd
36	HCBd	NEG	0.8	nd	nd	nd

*Coeliac disease (CD, patients 1–14), non-coeliac gluten sensitivity (NCGS, patients 15–31), and healthy control blood donors (HCBd, patients 32–36). EMA, anti-endomysial antibodies; tTG, anti-tissue TG antibodies; DGP, anti-deamidated gliadin peptide antibodies; AGA, anti-gliadin antibodies; IgA, immunoglobulin A; IgG, immunoglobulin G; pos+-- , weak positive; pos+ + -, positive; pos+ + + , strong positive; neg, negative; nd, not detectable.*



## Protein Profile Analysis by SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). Samples were treated with reducing sample buffer for 5 min at 95°C and then run on 15% SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 at room temperature and then destained in 10% (v/v) acetic acid. Gels were scanned and analyzed using the Bio-Rad Image Lab 4.0.1 Software.

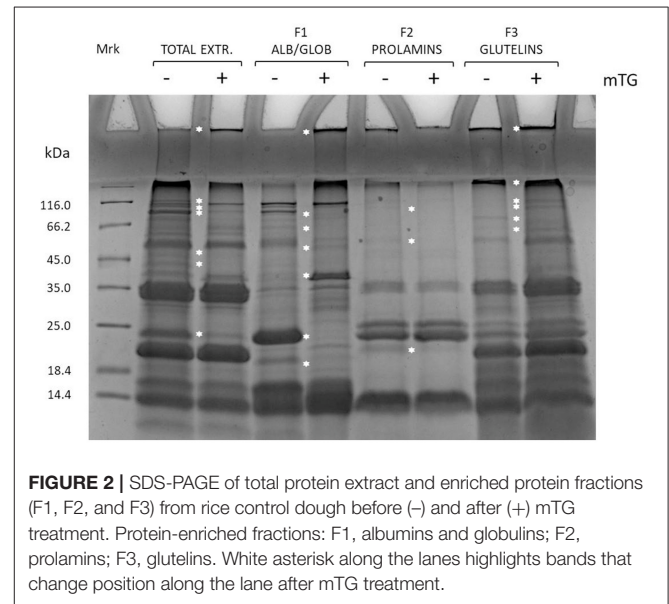
## Analysis of the IgG-Binding Capacity by ELISA

First, 96-well Maxisorp plates (Nalgene, Rochester, NY) were coated with 50  $\mu$ L of the protein sample (5  $\mu$ g/mL) in 150 mmol/L phosphate buffer saline (PBS) overnight at 4°C. The plate was washed twice with PBS and subsequently nonspecific binding sites were blocked with 200  $\mu$ L/well of 1% BSA in Tris buffer saline containing 0.05% Tween-20 (TBS-T), for 1 h at room temperature. Several serum dilutions (ranging from 1:250 to 1:2,500) were tested in order to identify the optimal number of antibodies for the development of the ELISA analysis. IgG-binding capacity was tested by the addition of 50  $\mu$ L/well of diluted patient and healthy control sera or pooled sera, respectively, and incubated overnight at 4°C. For each protein sample, blank controls were tested using 0.5% BSA in TBS-T instead of the diluted serum. After four washes, 50  $\mu$ L/well alkaline phosphatase-conjugated polyclonal anti-human IgG antibody (Thermo Fisher Scientific), diluted 1:1,000 in TBS-T containing 0.5% BSA, was added. After 1 h of incubation at 37°C and another one at 4°C, the plate was washed four times with TBS-T and developed with 50  $\mu$ L/well of substrate solution, consisting of 10 mmol/L p-nitrophenyl phosphate (PNPP) dissolved in alkaline substrate buffer pH 9.8 (9.7% diethanolamine and 1 mM MgCl<sub>2</sub>). The absorbance was recorded

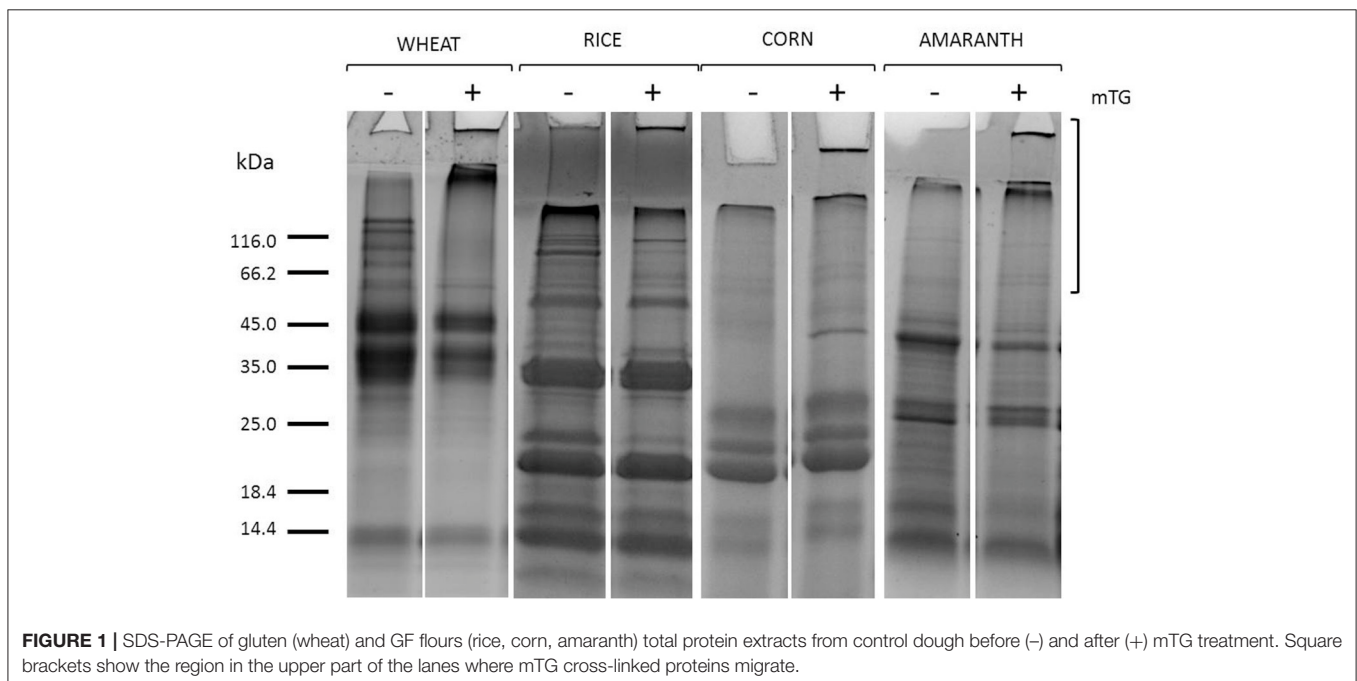
using a microplate ELISA reader at 405 nm (ref. 490 nm). The IgG-binding capacity of each protein sample was characterized by corresponding OD values after background subtraction. The ELISA protocol, coating conditions, reagent dilutions, buffers and incubation times were tested in preliminary experiments.

## Statistical Analysis

All data are reported as means  $\pm$  SD. Data were analyzed using GraphPad Prism by one-way ANOVA. Differences were considered significant when  $p < 0.05$  and very highly significant when  $p < 0.001$ .



**FIGURE 2 |** SDS-PAGE of total protein extract and enriched protein fractions (F1, F2, and F3) from rice control dough before (-) and after (+) mTG treatment. Protein-enriched fractions: F1, albumins and globulins; F2, prolamins; F3, glutelins. White asterisk along the lanes highlights bands that change position along the lane after mTG treatment.



**FIGURE 1 |** SDS-PAGE of gluten (wheat) and GF flours (rice, corn, amaranth) total protein extracts from control dough before (-) and after (+) mTG treatment. Square brackets show the region in the upper part of the lanes where mTG cross-linked proteins migrate.

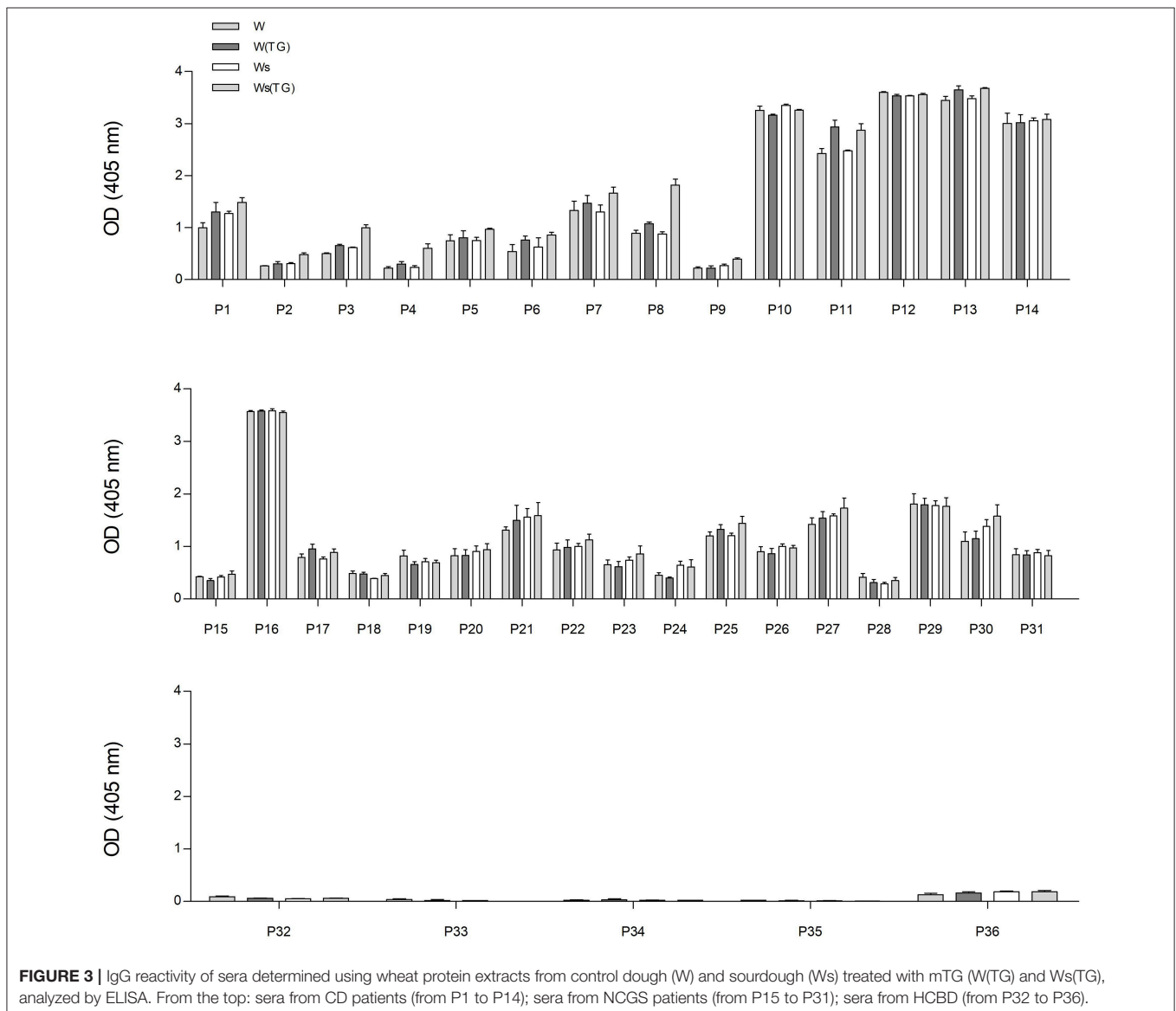
## RESULTS

### Cross-Linking Effect of mTG on Protein Extracts

The mTG protein cross-linking products were evaluated in control dough, after protein extraction and separation by SDS-PAGE. As shown in **Figure 1**, after mTG treatment, the protein profiles of gluten-containing dough and GF dough in total protein extracts consisted of the disappearance of some bands along the lanes and the accumulation of protein aggregates. Some protein aggregates were unable to enter the resolving gel, whereas a portion of crosslinked protein does not even enter the stacking gel. These results are the consequence of the cross-links between glutamine and lysine residues of protein substrates catalyzed by mTG (Scarnato et al., 2017).

The effects of enzyme treatment in the control dough were evaluated also on protein enriched fractions, as shown in **Figure 2**.

Fractions enriched in F1 and F3 were the main ones involved in protein aggregation because of mTG catalysis of cross-linked products. This has been revealed by the higher accumulation of proteins in the wells and in the running-stacking gel boundary regions in mTG-treated samples as compared with the non-treated ones. Moreover, along the lanes, some bands present in the non-treated sample disappeared in the treated ones, possibly because of protein aggregation to form high molecular weight products. This is particularly evident for the 24 kDa band present in F1 fraction (not treated with mTG) that disappeared when this fraction was treated with mTG. Similar results were also observed either in the other gluten-containing or the GF dough protein extracts



(total and enriched fractions) treated with mTG (data not shown). The effect of mTG on sourdough protein extracts from wheat and GF flours has been previously reported (Scarnato et al., 2016, 2017).

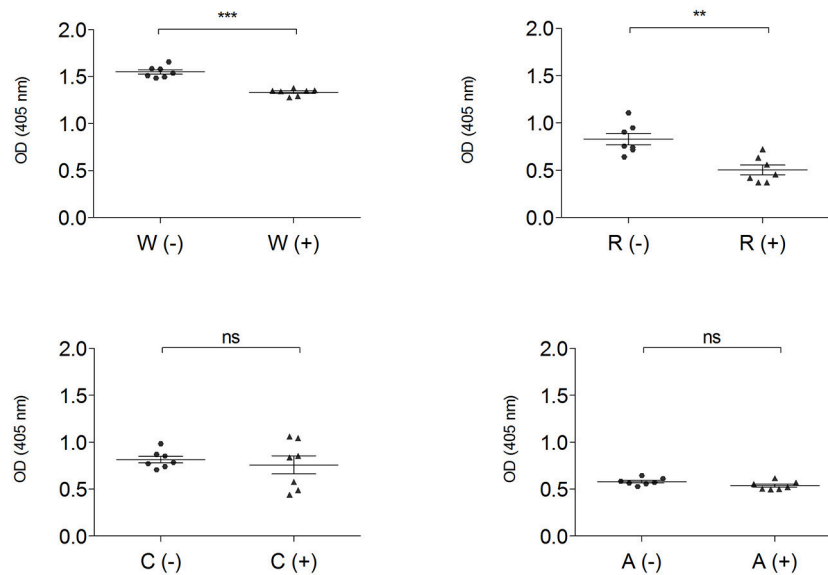
## mTG Effects on Flour Proteins Immunoreactivity

In order to analyze the immunoreactivity of gluten and GF proteins from control dough and sourdough, treated or not with mTG, total protein extracts and F1-, F2-, and F3-enriched fractions were analyzed by checking the IgG-binding capacity of sera from gluten-sensitive patients and healthy blood donors. Single serum IgG reactivity toward total wheat proteins extracted from dough (W) and sourdough (Ws) is shown in **Figure 3**.

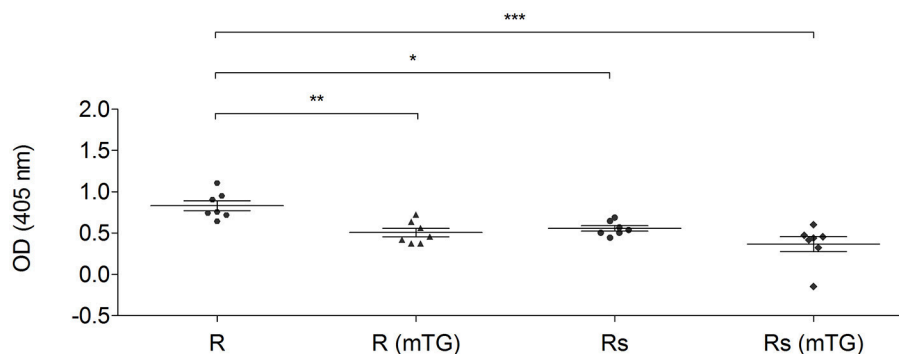
The IgG reactivity distribution of sera reflected the antibody titers of each individual patient. Sera from CD patients and NCGS P16 showed the highest immunoreactivity in terms of OD values, whereas HCBBD sera gave no or very low signals.

The graphs in **Figure 3** represent the IgG reaction of each individual serum used in the study. There are no significant differences in reactivity within each individual serum toward wheat proteins [all four types: total wheat extract (W), total extract treated with mTG (W(TG)), sourdough (Ws), sourdough + mTG (Ws(TG))].

From the data presented in **Figure 3**, it is possible to observe that the reactivity of the 36 patients among each other is extremely variable even inside categories of CD, NCGS patients. There is generally a higher IgG reactivity in the CD group, medium reactivity for the NCGS and nearly no reactivity in



**FIGURE 4** | IgG-binding capacity, expressed as OD value of gluten-containing dough and GF flour control dough before (-)  $\blacktriangle$  and after (+)  $\bullet$  mTG treatment using pooled CD patient' sera (P1–P14). Data are presented as mean values of 7 replicates with significance level: ns= not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . W, wheat; R, rice; C, corn; A, amaranth.



**FIGURE 5** | IgG-binding capacity of rice total protein extracts using a pool of sera from CD patients (P1–P14). The protein from rice dough and sourdough were treated with mTG. Data are reported as absorbance at 405 nm of sera diluted 1:250. Data are presented as mean values with significant level: \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

the healthy controls. This figure is associated with the **Table 1**, which describes the clinical diagnosis and serum antibodies level of patients.

The IgG-binding capacity of total protein extracts, before (-) and after (+) mTG treatment, was analyzed using CD patients' pooled sera in order to have a representative trend and to eliminate the variability factor among individuals; data of wheat and GF flours control dough are reported in **Figure 4**.

Enzymatic treatment did not change the immunoreactivity of total protein extracts both in corn and in amaranth, while in wheat ( $p < 0.001$ ) and rice doughs ( $p < 0.01$ ), mTG treatment significantly decreased the IgG-binding capacity. The OD values represent the level of IgG reactivity to any of the wheat, rice, corn and amaranth proteins present in dough extracts. A higher value indicates that more IgG antibodies against surface-exposed proteins of the flour sources are present in the sera.

The combined effect of mTG and sourdough on GF dough proteins was also analyzed using CD, NCGS, and HCBP pools of sera. Both biotechnological treatments (mTG and sourdough) influenced the immuno-recognition of the sera. As representative data, the total protein extracts from rice doughs, analyzed with the CD patients pool of sera, are shown in **Figure 5**.

mTG treatment significantly reduced ( $p < 0.01$ ) the specific antibody binding capacity of the protein derived from rice dough. Sourdough fermentation caused a reduction ( $p < 0.05$ ) of the immunoreaction. The statistical analysis did not show any significant differences when the Rs (mTG) sample was compared to the Rs and to R (mTG) samples. In fact, both treatments (sourdough and mTG) resulted in significant antigenicity reduction and the combined treatment did not lead to more decreased antigenicity (**Figure 5**).

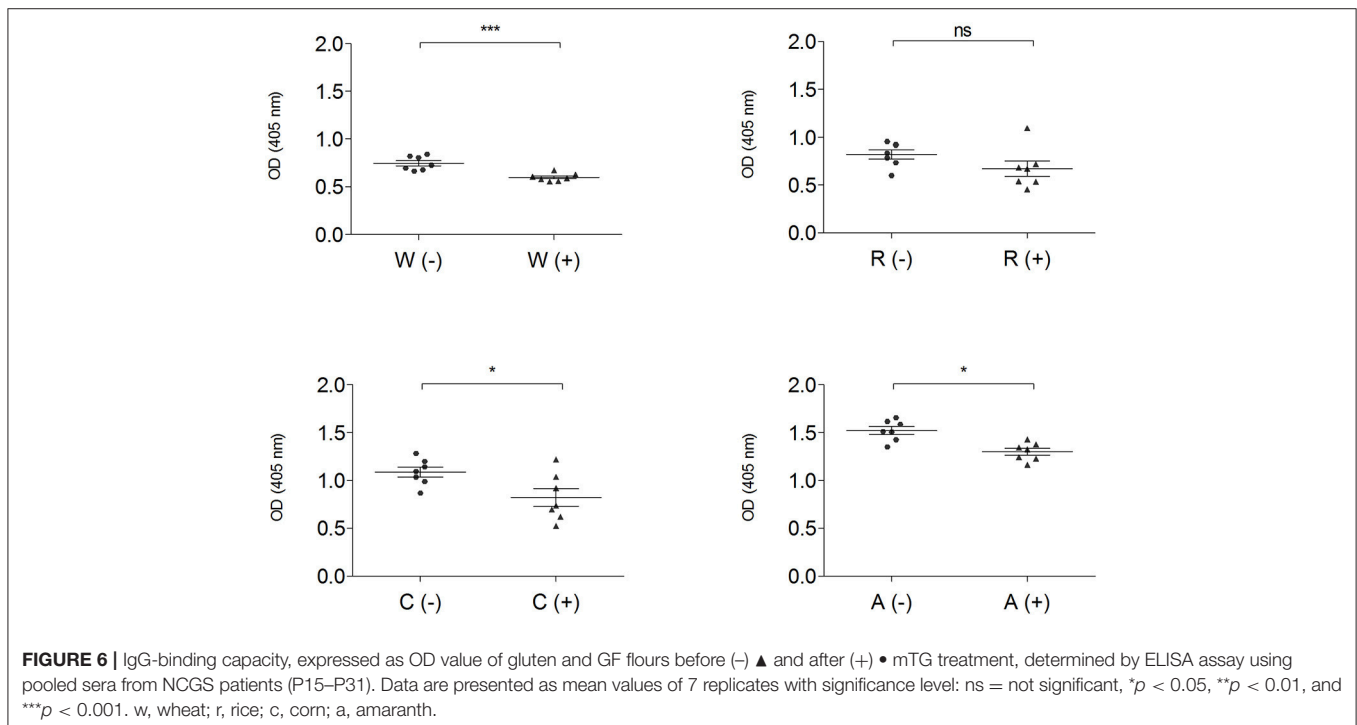
The IgG-binding capacity of total protein extracts from gluten-containing dough and GF dough was measured using pooled sera from NCGS patients (P15–P31) (**Figure 6**).

This analysis, performed both with wheat and GF doughs, supports the data obtained with CD sera; dough protein immunoreactivity did not increase after mTG treatment. On the contrary, when tested with NCGS sera, mTG treatment decreased immune recognition in doughs of wheat, corn and amaranth (**Figure 6**). To further evaluate protein IgG reactivity, control dough proteins were extracted by a sequential method using specific buffer solutions. IgG-binding capacity toward F1-, F2-, and F3-enriched fractions, treated or not with mTG, was tested in order to better identify which class of protein showed the highest signal when treated with sera from CD and NCGS patients.

Results indicate a different immunoreactivity of wheat dough compared to GF dough samples. In fact, wheat protein extracts, and in particular the F3 fraction, showed the highest immunoreactivity, both when tested with CD and NCGS sera. In all the GF samples, immunoreactivity of F2 and F3 fractions was very low and in amaranth F3 did not reach the detection limit of the method. In general, F1 of the GF samples was the only fraction showing considerable immunoreactivity (**Figure 7A**). In all samples, the CD patients' sera showed a higher immunoreactivity when compared to NCGS patients (**Figure 7**). Moreover, mTG treatment did not affect the IgG reactivity profile in any of the tested flour doughs.

## DISCUSSION

The lack of structure in bread dough is a difficult challenge while working with GF cereal products. In fact, GF products

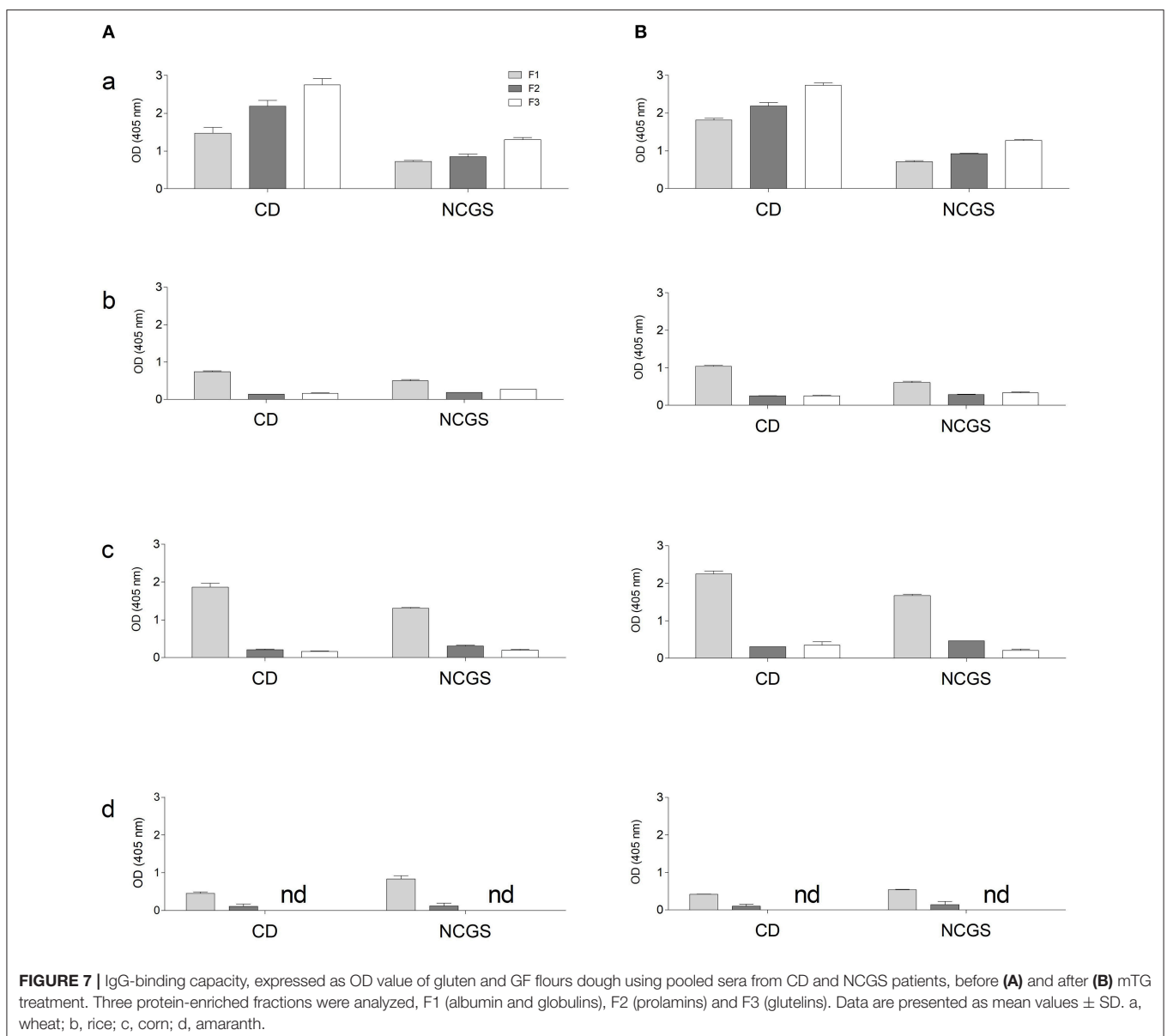




available on the market are often of low nutritional quality and poor taste. Without gluten, wide ranges of ingredients (i.e., hydrocolloids) are needed to obtain products with organoleptic features appreciated by consumers (Moreira et al., 2013). mTG is proposed as a biotechnological agent to improve the functional properties of structurally poor flour proteins as a processing aid, as it can induce structural protein modifications improving the features of the final product (Camolezi Gaspar and Pedroso de Góes-Favoni, 2015). In our earlier work, GF cereal doughs and sourdoughs made by using *Lactobacillus sanfranciscensis* and *Candida milleri* were subjected to enzymatic treatment by supplying mTG from *Streptomyces mobaraensis* in order to obtain doughs with an improved texture (Scarnato et al., 2016, 2017). However, the effect of mTG on the immunoreactivity of these doughs was not completely clarified. Therefore, this study was

undertaken in order to verify if the mTG reaction affected the immunoreactivity of the treated doughs. In fact, concerns were raised about the use of mTG for flour protein modification as human tissue TG is involved in gliadin deamidation, a key reaction in the etiology of CD. Deamidated gliadins are known to increase immunoreactivity to gluten peptides in CD patients (Sollid, 2000). Moreover, Gerrard and Sutton (2005) suggested that further research was needed to assess this possibility and recommended that TG should not be used in bakery products until this issue is resolved.

Previous studies showed that mTG in combination with sourdough exhibited a positive and synergistic effect by which the excessive hardness and chewiness caused by mTG were counteracted by the sourdough. On the other hand, the protein-aggregating effect of mTG compensated for the



proteolytic action of sourdough on protein substrates, which reduces the viscoelastic properties of bread. Data showed that the gluten fraction was the main fraction involved in these cross-links, but in GF flours, mTG was able to exert its action also on the F1 (Albumin and Globulin)-enriched fraction. When mTG activity was checked by microplate assay (while incorporating biotin-cadaverine (BC) on protein substrate immobilized on a microplate), F2 was the main fraction involved in the incorporation of BC, followed by F1-enriched fraction in lentil and amaranth and by F3 (Glutelin)-enriched fraction in rice and corn. The use of sourdough combined with mTG showed a synergistic beneficial effect on bread characteristics, with improved bread rheological features, aroma profile and shelf-life of the baked product. The excessive hardness and chewiness of bread caused by increasing concentrations of mTG were counteracted by the addition of a proper amount of sourdough. On the other hand, the degradative action of sourdough on protein substrates, which reduced the viscoelastic properties of bread, was compensated by the protein-aggregating effect of mTG (Scarnato et al., 2016, 2017).

The aim of the present research was to investigate if the protein modification catalyzed by mTG could affect the immunological features of dough proteins, previously studied for their rheological and organoleptic properties. Data showed that mTG treatment in both gluten and GF flours did not cause a significant increase of IgG-binding capacity. These results provide a perspective in research on GF products, suggesting the possible use of mTG as a biotechnological agent able to create innovative products; its action does not alter the IgG-binding epitopes on substrate proteins.

Under the experimental conditions of our study, mTG protein cross-linking did not affect antibody-binding capacity, thus corroborating results from previous studies. For example, no immunological changes of gliadin extract from pasta dough treated with mTG using the sera of CD patients was observed (Ruh et al., 2014). Interestingly, other data showed that cross-linked gluten flour had a lower immunoreactivity in a rabbit model system, suggesting that the lower deamidation rate of mTG relative to mammalian TGs, together with the cross-linking of gluten peptides, might potentially reduce this risk (Leszczynska et al., 2006). Moreover, transamidation of wheat flour with a food-grade enzyme and an appropriate amine donor can be used to block T cell-mediated gliadin activity and to prevent cereal toxicity (Gianfrani et al., 2007). Immunoblotting using monoclonal antibodies specific to unmodified and/or deamidated gliadin showed no differences between control bread and bread obtained after treatment of the dough with mTG.

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According to the authors, the concentrations of mTG used in wheat bread preparation do not lead to detectable amounts of deamidated gliadin (Heil et al., 2017).

On the other hand, Berti et al. (2007) demonstrated that mTG-deamidated gliadins increase the IgA antibody reactivity of CD patients with respect to control gliadins (Berti et al., 2007). Others reported an increased immunoreactivity of a CD serum pool to gliadin from bread treated with mTG (Gerrard and Sutton, 2005; Cabrera-Chávez et al., 2008). Recently, Torsten and Aaron (2018) hypothesized that mTG used in food preparation could favor celiac disease initiation and progress. As TGs are also present in plants (Del Duca et al., 2000, 2010; Skovbjerg et al., 2002; Serafini-Fracassini et al., 2009), plant-derived food could be another source for TGs that might reach the reach intestinal tract where these TGs (food derived ones and mTG from microbiota) could play a pathogenic role. This last hypothesis is suggestive but, to our knowledge, not supported by solid experimental evidence.

To settle the question, it has been suggested to test new products by applying the immunoreactivity assay using the sera of CD patients (Cabrera-Chávez et al., 2008). This simple and reliable test could be an easy way to evaluate ingredients and procedures to obtain new GF products and to identify potentially unsafe products. Moreover, further investigations using mouse models could be useful to assess the immunogenicity of mTG doughs and sourdoughs.

In summary, by following the experimental procedure reported in this paper, treatment of gluten and GF flour doughs with mTG leads to an increase in cross-links but does not lead to significant changes in the IgG binding reactivity of the protein extracts with sera from either CD or NCGS patients' sera.

## AUTHOR CONTRIBUTIONS

LS, GG, and SD conceived the research and experiment design. LS performed the experimental work. UV, RD, and GC collected patient's sera. RL developed and selected the microbial strains for sourdough. All authors contributed in result discussion, manuscript drafting and revision.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Bioproduction of the Recombinant Sweet Protein Thaumatin: Current State of the Art and Perspectives

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There is currently a worldwide trend to reduce sugar consumption. This trend is mostly met by the use of artificial non-nutritive sweeteners. However, these sweeteners have also been proven to have adverse health effects such as dizziness, headaches, gastrointestinal issues, and mood changes for aspartame. One of the solutions lies in the commercialization of sweet proteins, which are not associated with adverse health effects. Of these proteins, thaumatin is one of the most studied and most promising alternatives for sugars and artificial sweeteners. Since the natural production of these proteins is often too expensive, biochemical production methods are currently under investigation. With these methods, recombinant DNA technology is used for the production of sweet proteins in a host organism. The most promising host known today is the methylotrophic yeast, *Pichia pastoris*. This yeast has a tightly regulated methanol-induced promoter, allowing a good control over the recombinant protein production. Great efforts have been undertaken for improving the yields and purities of thaumatin productions, but a further optimization is still desired. This review focuses on (i) the motivation for using and producing sweet proteins, (ii) the properties and history of thaumatin, (iii) the production of recombinant sweet proteins, and (iv) future possibilities for process optimization based on a systems biology approach.

**Keywords:** thaumatin, natural sweetener, *Pichia pastoris*, sweet protein, recombinant proteins, systems biology

## INTRODUCTION

Food manufacturers are in constant search for alternative sweeteners for their products in accordance with the change of consumer perception on the intake of sucrose. Most of the times, the development of these alternatives poses certain difficulties in terms of meeting all the requirements for replacing sucrose. Due to the complex combination of properties existing within sucrose in terms of its taste and texture characteristics, it is often seen as a challenging task to replace it with a low-calorie or non-caloric sweetener.

## Sugar

Sugars are extensively used ingredients in numerous applications especially in food and pharmaceuticals. It is a disaccharide which consists of one unit of glucose and one of fructose linked

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by glycosidic bonds and gets easily hydrolyzed into these simple sugars upon digestion. Due to the increased demand for sucrose, the production of these ingredients has elevated over the years and lingers as an important part of the diet worldwide.

The fragmentation of sugar into constituent monosaccharides occurs within the small intestine in the presence of the digestive enzymes and eventually gets transported into the bloodstream from the intestines. This leads to the elevation of blood glucose level until it gets transported by insulin (Pfeiffer et al., 2010). The breaking down of sugar is associated with its complexity, i.e., the more complex the sugar is, the higher the energy and longer the time required for it to break the linkages. Therefore, the elevation of the blood glucose level is dependent on the type of sugar consumed. Due to this effect on the blood glucose level, sugars can contribute to various adverse health effects. Bray and Popkin (2014) elaborate on such an outcome in their study by utilizing the results from clinical trials and meta-analyses. From their analyses and trials, it was observed that the consumption of sugar-sweetened beverages (SSBs) induced the risk of diabetes, metabolic syndrome and cardiovascular diseases and a reduction of weight gain could be achieved by minimizing the intake of these drinks.

Furthermore, several other studies also point toward the fact that high intake of sugars can instigate risk factors such as high blood pressure (He and MacGregor, 2015) and cardiovascular diseases (Johnson et al., 2009). Due to the high amount of energy that sugars provide to the body, they contribute to obesity as well. According to the findings of NCD-RisC (NCD Risk Factor Collaboration, 2016), it is estimated that obesity will be noticeably prevalent among adults worldwide with a possibility to increase further by 2025.

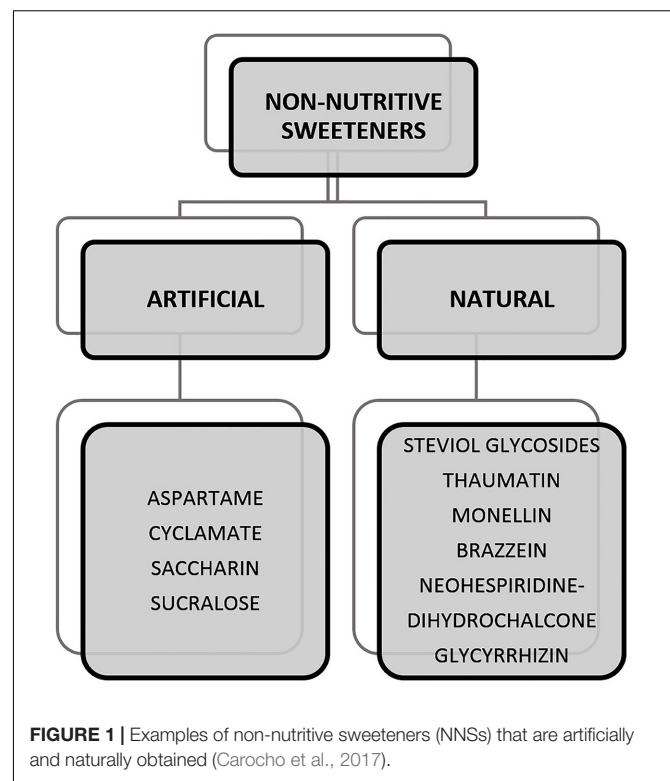
As a food ingredient, table sugar has played a paramount role until its value began to subside due to the public awareness which surfaced over the past years. This in turn triggered the development of alternative sweeteners that could effectively produce a similar effect in applications but have less impact on the overall health. The decrease in consumption of table sugar is evident from the data acquired on the changing trends in the consumption of low-calorie sweeteners (Sylvetsky and Rother, 2016). Moreover, the World Health Organization (WHO) now emphasizes the necessity for reducing added sugars in the diet and hence, recommends an intake of sugar less than 5% of the total caloric intake<sup>1</sup>.

## Sugar Replacement

The awareness on the health issues among the acknowledged has imposed the need for alternative solutions that have less impact on human health. Since the 1800s many sweeteners, both natural and artificial have been identified for various applications. The potency of these sweeteners is most often higher than that of sucrose.

Sweeteners can be classified as caloric, low-calorie, and non-caloric. The caloric sweeteners are also known as nutritive sweeteners which provide energy in the form of carbohydrates. These can be seen naturally occurring in foods such as fructose

<sup>1</sup>[apps.who.int/iris/bitstream/10665/149782/1/9789241549028\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/149782/1/9789241549028_eng.pdf)



in fruits or added to foods such as sucrose to drinks. This class of sugars also includes sugar alcohols such as sorbitol, lactitol, xylitol, mannitol, erythritol, trehalose, and maltitol. They provide calories which possibly contribute to health conditions on consumption in high quantities. In the case of sugar alcohols, consumption can also lead to other effects, i.e., irritable Bowel syndrome (IBS) and abnormal flatulence (Tuck et al., 2014; Yao et al., 2014). Specifically, erythritol, has the tendency to cause laxative effects when consumed in high quantities (Rzechonek et al., 2018). In this context, the rise of non-nutritive sweeteners (NNS) as an alternative ingredient surged within the market. **Figure 1** provides a list of NNS that are artificially and naturally obtained and have been studied widely. Some of the NNS that are approved for use in food includes sucralose, aspartame, saccharin, acesulfame K, neotame, advantame, steviol glycosides, and luohanguo (monk fruit) extract out of which, the latter two are naturally derived NNS<sup>2</sup>. A more detailed discussion on artificial and natural NNS is provided in Section “Artificial Sweeteners” and “Natural Sweeteners.”

The selected alternative sweetener should withhold certain important properties that make it ideal for similar applications as sucrose. For instance, Bakal (1986) explains the prominent characteristics that an alternative sweetener is expected to hold when used as a substitute for sucrose. The author explains that in addition to being as sweet as sucrose, it must also possess similar functional and physiological properties. Sweetness factor is an inevitable criterion when finding substitutes for sucrose

<sup>2</sup><http://www.fda.gov/food/ingredientpackaginglabeling/foodadditivesingredients/ucm397725.htm>

especially because the potency value of these sweeteners is defined with respect to it. Besides this, the substitutes are also expected to provide a sensory profile which is similar to that of sucrose. However, some authors claim it to be very challenging for acquiring similar taste characteristics to the distinctive taste profile of sucrose (Larson-Powers and Pangborn, 1978; Palazzo and Bolini, 2013). Sucrose also plays a paramount role in delivering specific properties such as structure, texture, and flavor (Koeferli et al., 1996; Pareyt et al., 2009) in various applications making it further distinctive in nature. Moreover, Hough (1997) emphasizes properties such as solubility, chemical and thermal stability, compatibility with production and applications, ease of production, non-toxicity and being inexpensive.

## Artificial Sweeteners

Artificial sweeteners can be described as non-caloric or low-calorie sweeteners because they provide very few calories when compared with sucrose due to lesser absorption by the digestive system. Unlike the caloric or nutritive sweeteners, they provide no calories or hardly any calories but possess a much higher sweetness than sucrose. These intense sweeteners are usually mixed with bulking agents such as polydextrose and maltodextrin to enhance their applicability. Although the influence of artificial sweeteners on health aspects has been discussed widely in the past, there are still some uncertainties revolving around these sweeteners in terms of usage limitations (Marti et al., 2008). Examples of this class are acesulfame-K, aspartame, neotame, saccharin and sucralose, which are approved by the United States Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA). The sweetness index of these sweeteners is included along with their E numbers in **Table 1**.

Out of these artificial sweeteners, aspartame is a prevalent ingredient around the globe and is used in a considerable number of food products (Myers, 2007). Despite the benefit of using it as an alternative to sucrose in numerous applications, the safety aspects still remain controversial. For instance, some health effects such as dizziness, headaches, gastrointestinal issues, and mood changes are associated with aspartame. These health effects are elaborated in the study of Whitehouse et al. (2008). Ferland et al. (2007) investigated the effect of this sweetener on the glucose and insulin levels in blood

plasma. The target group selected for the study were men diagnosed with type 2 diabetes and the influence of aspartame was assessed during acute exercise. During their investigation, it was noticed that the consumption of aspartame led to an increase in glucose and insulin levels similar to that of sucrose. Pearlman et al. (2017) emphasizes that the artificial sweeteners have an impact on host microbiome, gut-brain axis, glucose homeostasis, energy consumption, overall weight gain, and body adiposity that is evident through the profound amount of available data. With regard to the microbiota, a study conducted by Suez et al. (2014) claims that the usage of non-caloric artificial sweeteners can boost the risk of glucose intolerance.

Apart from this, several other studies on artificial sweeteners have developed severe concerns among the consumers regarding its usage. For instance, these artificial sweeteners can enter the environment and undergo degradation thereby resulting in toxic products. The authors Kokotou et al. (2012) summarize the impact of these ingredients as environmental pollutants. In their review article, they elaborate on the findings related to the environmental impact of artificial sweeteners along with the available methodologies that can be incorporated for detection of the trace compounds. While NNS are widespread today for numerous applications, with respect to the approval from FDA and other studies, the safety aspects regarding their regular usage remain uncertain<sup>3</sup>. It is therefore not surprising that the World Health Organization (2015) predicts an increase in the production of natural alternatives based on the shift in consumer preferences toward more natural products.

## Natural Sweeteners

In this context, the plant-derived sweeteners such as stevioside, glycyrrhizin, osladin, etc. come into the limelight with the added advantage of not imposing any health issues. For example, stevia which is a natural sugar substitute was not seen to increase the blood glucose levels compared to sucrose with the findings of Anton et al. (2010). During this study, the effect due to the intake of stevia, aspartame, and sucrose were compared. The study concluded that stevia reduced blood insulin levels significantly compared to both sucrose and aspartame. This demonstrates the health benefit of a natural sweetener compared to an artificial sweetener.

Apart from the previously mentioned sweeteners, there are several sweet compounds that are found in nature belonging to three classes namely terpenoids, flavonoids, and proteins (Kinghorn and Compadre, 2001; Fry, 2012). Most proteins do not necessarily elicit any sweet taste and flavor. However, this is not the case with the sweet proteins derived naturally from plants that are grown in tropical regions of mostly Africa and Asia. These naturally derived proteins are very sweet, i.e., even 100–1000 times sweeter than table sugar or sucrose on a mass basis. Such sweeteners which are low-calorie or non-caloric in nature can be used to substitute sucrose in sugar-based food and drinks thereby posing a solution for those who are prone

**TABLE 1** | The major artificial sweeteners used in the food and pharmaceutical industry (Lindley, 2012; Chattopadhyay et al., 2014).

Sweetener	Sweetness index	E Number
Acesulfame	200 x	E 950
Aspartame	180–200 x	E 951
Cyclamate	30–50 x	E 952
Saccharin	300 x	E 954
Sucralose	600 x	E 955
Alitame	2000 x	E 956
Neotame	7,000–13,000 x	E 961
Advantame	20,000 x	E 969

<sup>3</sup><http://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm397725.htm>

**TABLE 2** | Comparison on the characteristics of different sweet proteins.

Sweet protein	Sweetness (weight basis)	Amino-acids	Properties	Natural Source	Bioproduction host
Thaumatococcoside	3000 x	207	<ul style="list-style-type: none"> <li>Taste slightly differs from sucrose</li> <li>used as flavor modifier</li> <li>Stable at 120°C; withstand pasteurization and UHT process</li> <li>EU approved (E957)</li> </ul>	<i>Thaumatococcus daniellii</i> Benth	Bacteria <sup>1,2,3</sup> yeast <sup>4,5,6</sup> Fungi <sup>7,8</sup> Transgenic plant <sup>9,10</sup>
Monellin	3000 x	45 (A chain)/50 (B chain)	<ul style="list-style-type: none"> <li>Intense sweetness</li> <li>Loss of sweetness above 50°C</li> </ul>	<i>Dioscoreophyllum cumminsii</i> Diels	<i>E. coli</i> <sup>11</sup> Yeast <sup>12</sup> Transgenic plant <sup>13</sup>
Brazzein	2000 x	54	<ul style="list-style-type: none"> <li>Heat and pH stable; 100°C for 2 h</li> <li>Tastes similar to sucrose</li> <li>Shorter aftertaste</li> <li>Water soluble</li> <li>Works well with stevia</li> </ul>	<i>Pentadiplandra brazzeana</i> Baillon	<i>S. cerevisiae</i> <sup>14</sup> <i>E. coli</i> <sup>15</sup>
Neoculin/curculin	550 x	114	<ul style="list-style-type: none"> <li>Degrade at 50°C</li> </ul>	<i>Curculigo latifolia</i>	<i>E. coli</i> <sup>16</sup> <i>Aspergillus oryzae</i> <sup>17</sup>
Mabinlin	100 x	33 (A chain) 72 (B chain)	<ul style="list-style-type: none"> <li>4 Variants</li> <li>Mab II unchanged at 80°C for 2 hrs</li> <li>1, 3, and 4 not stable</li> </ul>	<i>Capparis masakai</i> Levi	<i>E. coli</i> <sup>18</sup> Cultured plant cells <sup>19</sup>
Pentadin	500 x	54	<ul style="list-style-type: none"> <li>Rapid loss of sweetness</li> </ul>	<i>Pentadiplandra brazzeana</i> Baillon	–
Miraculin	N. A	191	<ul style="list-style-type: none"> <li>Modifies sour taste into sweet taste</li> </ul>	<i>Richadella dulcifica</i>	<i>E. coli</i> <sup>20</sup> Transgenic plants <sup>21</sup>

<sup>1</sup>(Faus et al., 1996); <sup>2</sup>(Illingworth et al., 1988); <sup>3</sup>(Illingworth et al., 1989); <sup>4</sup>(Edens et al., 1984); <sup>5</sup>(Edens and van der Wel, 1985); <sup>6</sup>(Cregg et al., 2000); <sup>7</sup>(Hahm and Batt, 1990); <sup>8</sup>(Faus et al., 1998); <sup>9</sup>(Witty, 1990); <sup>10</sup>(Bartoszewski et al., 2003); <sup>11</sup>(Chen et al., 2005); <sup>12</sup>(Kim and Lim, 1996); <sup>13</sup>(Peñarubia et al., 1992); <sup>14</sup>(Guan et al., 1995); <sup>15</sup>(Assadi-Porter et al., 2000); <sup>16</sup>(Suzuki et al., 2004); <sup>17</sup>(Nakajima et al., 2006); <sup>18</sup>(Gu et al., 2015); <sup>19</sup>(Xiong and Sun, 1996); <sup>20</sup>(Kurihara, 1994); <sup>21</sup>(Sun et al., 2006).

to diabetics or obesity. They come with the advantage of not imposing significant health concerns compared to the previously mentioned artificial sweeteners.

Until now, eight sweet proteins are identified namely, thaumatococcoside (Van der Wel and Loeve, 1972), monellin (Inglett and May, 1969), mabinlin (Liu et al., 1993), lysosyme (Maehashi and Udaka, 1998), pentadin (Faus, 2000), brazzein (Ming and Hellekant, 1994), curculin (Harada et al., 1994), and miraculin (Takahashi et al., 1990). These proteins are isolated mostly from tropical plants with an exception for the protein lysozyme, which is derived from egg whites. The research in sweet-proteins has been ongoing for several years now and some of the major characteristics of each protein have been studied over the years. The most important characteristics of these proteins are summarized in **Table 2**. Of these eight proteins, thaumatococcoside is the most developed, commercialized and regulated sweet protein (García-Almeida et al., 2018).

One of the most crucial factors to take into account while considering sugar replacers is the sweetness. The sweetness factor is measured with reference to sucrose. Sweetness perception occurs when sugar dissolves with the saliva and bind with the receptors on the tongue. Therefore, humans perceive sweet taste through the T1R2–T1R3 receptor which are heterodimers belonging to the family of G-protein-coupled receptors (GPCRs) (Margolskee, 2001; Nelson et al., 2001; Li et al., 2002). These receptors which have several binding sites (Vigues et al., 2008) get activated when the compounds that elicit sweet taste bind to it. However, the binding property for each sweetener is different and hence, all the sweeteners do not necessarily bind at the same sites (Fernstrom et al., 2012). This leads to the varying perception of sweetness of the

different proteins. **Table 3** lists some of the most important properties of bulk Thaumatococcoside.

## Review Outline

The increasing awareness among the public over the adverse effects of sucrose and artificial sweeteners is creating opportunities for the exploration of naturally derived alternatives, such as plant-derived sweeteners. In this review, the advances in the production of the sweet-protein thaumatococcoside will be discussed. Section “Thaumatococcoside” summarizes the current relevance of thaumatococcoside based on its characteristics and possible applications. Also, a detailing will be done on the aspects related to the production of this protein via the various routes that have been studied. Section “Bioproduction” focuses on the bioproduction aspects involved in the production of thaumatococcoside with a focus given to the selection of strains, DNA construction for protein expression, production process and downstream processes. The final section summarizes on the possibility to

**TABLE 3** | Properties of bulk thaumatococcoside.

Parameter	Specifications
Visual appearance	Yellow to light tan powder
Solubility (water)	600 mg/mL
pH	2.7–6.0
Stability (dry concentrate at <10°C)	>6 months
Minimum total thaumatococcoside content	≥98% HPLC

[www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticInventory/ucm592042.pdf](http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticInventory/ucm592042.pdf).

integrate knowledge from systems biology for the optimization of thaumatin bioproduction.

## THAUMATIN

Thaumatococcus, a sweetener desired in the market today, is naturally derived from the fruit arils of a tropically grown plant called *Thaumatococcus daniellii* (Benth) belonging to the family Marantaceae (Kant, 2005). *Thaumatococcus daniellii* is a large flowering herb which can grow up to 4 m high and is commonly found in the rainforests of West Africa ranging from Sierra Leone to the Democratic Republic of Congo. The fruit was called 'katemfe' or 'miraculous fruit of Sudan' (Daniell, 1855; Inglett and May, 1968; Van der Wel, 1974). They are also known as miracle fruit, miracle berry (Wiersema and León, 1999), Yoruba soft cane and African serendipity berry.

### Characterization of *Thaumatococcus*

The sweetness potency of thaumatin was first elucidated by a British surgeon, Daniell (1855). It elicits a sweetness 100,000 times higher than that of sucrose on a molar basis even at a low concentration of 50 nM. It is a caloric sweetener; however, it has a negligible impact at the level of concentration used within applications. It consists of a single-chain of 207 amino acid residues and has a relative molecular mass of 22 kDa (Van der Wel and Loeve, 1972). The stability of the protein is dependent on the matrix. For instance, the sweetness of the protein is retained when boiling at a pH below 5.5 for 1 h. It has even been observed that at these pH values the sweetener is stable during heat processes such as pasteurization, canning, baking and ultra-high-temperature processing (Gibbs et al., 1996; Lord, 2007). However, when heated above 70°C at a pH of 7.0, aggregation and loss of sweetness were observed (Mcpherson and Weickmann, 1990; Kaneko and Kitabatake, 1999). The loss of sweetness can be associated with heat denaturation or breakage of disulfide bridges (Higginbotham, 1979) within the protein. The heat and acid stability of the thaumatin molecule is due to the tertiary structure which is stabilized by the eight disulfide bridges (Van der Wel and Ledebauer, 1989).

It is important to know that different forms of thaumatin have been identified. The amino acid sequences of the two main forms, thaumatins I and II were originally reported by Iyengar et al. (1979) and Edens et al. (1982). A correction was made to the reported sequence for thaumatin I by Kaneko (2001). The two sequences are presented in **Figure 2**. Both forms have been expressed in microbial hosts. When studying the thaumatin sequences, Lee et al. (1988) assigned the two prominent variants as thaumatin A and thaumatin B. Thaumatin A later on appeared to be the same form as thaumatin I.

Numerous novel sweeteners have been introduced in the market that are expected to play as a substitute for sucrose by possessing similar sensorial properties as well as being safe. Prior to the introduction of these substitutes in the market, the laws and directives regulated by the authorities such as the FDA and EFSA must be followed. However, the rules and regulations differ throughout the world, which makes the

immediate introduction of these ingredients into the market more difficult. For those that are approved to be used, the regulatory bodies such as FDA, Scientific Committee for Food of the European Commission (SCF), or Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO have provided an acceptable daily intake. Considering the health aspects related to thaumatin, it does not instigate any tooth decay and can be suitable for the diabetic, unlike artificial sweeteners (Kingham et al., 1998). Moreover, the metabolism of this sweetener is analogous to other dietary proteins. The study conducted by Hsu et al. (1977) shows that digestion of thaumatin occurs more rapidly than egg albumin. Additionally, several studies involving the safety aspects of thaumatin indicate that the sweetener does not cause any allergenicity or toxicity. Numerous studies have been conducted to assess the toxicity of thaumatin. For instance, the Joint FAO/WHO Expert Committee on Food Additives, Food and Agriculture Organization of the United Nations & World Health Organization (1986) report claims that the protein is free from any toxic, genotoxic, or teratogenic effect. There is substantial evidence from various research, demonstrating that thaumatin is not an allergen to oral mucosa (MacLeod et al., unpublished) and on other treatment associated allergic effects (Eaton et al., 1981). The authors Higginbotham et al. (1983) also denote that thaumatin does not cause any hazardous effect when used as a flavor modifier or partial sweetener within a specific level of consumption. The safety of this protein was evaluated by SCF and JECFA who concluded that thaumatin can be considered as an acceptable ingredient for use (European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources Added to Food, 2015). This sweet protein is approved within the European Union since 1984 (E957) under Annex II of Regulation (EC) No. 1333/2008 (European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources Added to Food, 2015) and possesses GRAS (Generally Regarded as Safe) approval in the United States (FEMA GRAS Number 3732). It was approved in Great Britain in 1983 to be used in food and pharmaceuticals with an exception for baby foods. It is also allowed as a flavor enhancer and high-intensity sweetener within several countries (Zemanek and Wasserman, 1995). The FEEDAP (2011) also signals the safety of the protein for animals and approves its use as an additive within a level of 1 to 5 mg/kg. Some of the characteristics of thaumatin are listed in **Table 4**.

Apart from being a low-calorie sweetener, thaumatin can also act as a flavor modifier in food applications. Hence, such unique properties of this sweet-protein make it an attractive ingredient for the food industry. The major applications of thaumatin are seen as an additive in chewing gum, dairy, pet foods, and animal feeds (Smith and Hong-Shum, 2011). It also qualifies as a sweetener in other food products such as ice-creams and sweets within a permitted level of 50 mg/kg. In soft drinks and dairy products, it is mostly utilized as a flavor enhancer within a limit of 0.5 mg/L and 5 mg/kg respectively (Mortensen, 2006). The application of thaumatin is not just limited to imparting sweetness in products but also enhancing flavor and masking undesirable notes in food and pharmaceuticals (Etheridge, 1994). The onset of sweetness for this protein is slower and often results in a licorice aftertaste. Due



(I)				
10	20	30	40	50
MAATTCFFFL	FPFLLLLLTL	RAATFEIVNR	CSYTVWAAAS	KGDAALDAGG
60	70	80	90	100
RQLNSGESWT	INVEPGTNGG	KIWARDTCYF	DDSGSGICKT	GDCGGLLRCK
110	120	130	140	150
RFGRPPTTLA	EFSLNQYGKD	YIDISNIKGF	NVPMDFSPTT	RGCRGVRCAA
160	170	180	190	200
DIVGQCPAKL	KAPGGGCNDA	CTVFQTSEYC	CTTGKCGPTE	YSRFFKRLCP
210	220	230		
DAFSYVLDKP	TTVTCPGSSN	YRVTFCTAL	ELEDE	

(II)				
10	20	30	40	50
MAATTCFFFL	FPFLLLLLTL	RAATFEIVNR	CSYTVWAAAS	KGDAALDAGG
60	70	80	90	100
RQLNSGESWT	INVEPGTKGG	KIWARDTCYF	DDSGRGICRT	GDCGGLLQCK
110	120	130	140	150
RFGRPPTTLA	EFSLNQYGKD	YIDISNIKGF	NVPMDFSPTT	RGCRGVRCAA
160	170	180	190	200
DIVGQCPAKL	KAPGGGCNDA	CTVFQTSEYC	CTTGKCGPTE	YSRFFKRLCP
210	220	230		
DAFSYVLDKP	TTVTCPGSSN	YRVTFCTAL	ELEDE	

**FIGURE 2** | Amino acid sequence of the two main forms, thaumatin I and thaumatin II www.uniprot.org. Blue characters denote the signal peptide and red characters denote the differences in the sequences at four positions.

to this residual effect, thaumatin is often applied in combination with other sugar substitutes.

## History of Thaumatin Production

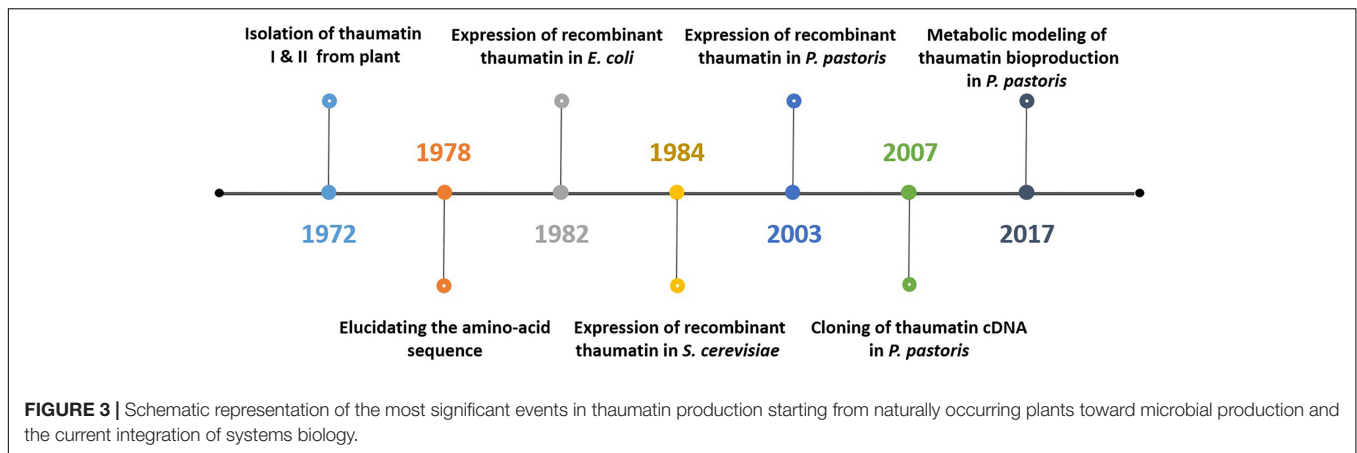
Bioprocessing plays a significant role in biotechnology. The advancement of molecular biology and recombinant DNA technology facilitates the production of human proteins through heterologous expression within microorganisms. For instance, human insulin, which is important to treat diabetes mellitus, was initially purified from bovine and porcine pancreas extracts.

**TABLE 4** | Recombinant thaumatin produced in different organisms.

Organism	Promoter	Secretion	Yield	Study
<i>E. coli</i>	Tryptophan/lactose	No	Low	Edens et al., 1982
<i>S. cerevisiae</i>	3-Phosphoglycerate	No	Low	Lee et al., 1988
<i>B. subtilis</i>	A-Amylase	Yes	1 mg/L	Illingworth et al., 1988
<i>S. lividans</i>	B-Galactosidase	Yes	0.2 mg/L	Illingworth et al., 1989
<i>A. awamori</i>	Glucoamylase	Yes	5–7 mg/L	Faus et al., 1998

The economic factor associated with its production combined with the immune responses caused in patients due to the animal insulin has instigated the implementation of heterologous expression using *Escherichia coli* (Jozala et al., 2016). The series of activities involved in the production of thaumatin follows a similar trend. **Figure 3** schematically represents how the production of thaumatin evolved from the traditional plant extraction to a more relevant microbial production today.

Initially, thaumatin was produced in 1972 by van der Wel and Loeve from the fruits of *Thaumatococcus danielli* through aqueous extraction. During this study, the authors examined the protein content from the fruit at various maturation stages and compared the amount of different forms of thaumatin such as thaumatins I and II. Both the forms were found to be similar with respect to their amino acid sequences. It was elucidated from the study that they have the same N-terminal amino acid alanine and molecular weights of  $21000 \pm 600$  and  $20400 \pm 600$  Da. Their findings also included the relation between the protein content in the fruit and the region of cultivation. During the study, a total of 4900 mg of extract per 2700 g of fruit was obtained (0.18%). The



sweetness of the aqueous solution of thaumatin obtained during the experiment was seen to decrease when heated above 75°C and also at a pH below 2.5 at room temperature. Korver et al. (1973) further investigated these two main forms of thaumatin. The study was to foresee the conformational changes undergone by the protein due to heat denaturation. It concluded that the loss of sweetness of the protein during the application of heat was basically due to the irreversible heat-induced conformational transition. The primary structure of thaumatin was unraveled by Iyengar et al. (1979) to gain a better understanding of the sweetness mechanism of the protein.

The product attained upon aqueous extraction from the Katemfe fruit was first found as a mixture of proteins. In the following year thaumatin I was crystallized from which the physical attributes and diffraction data of the crystals were attained (van der Wel et al., 1975). Although the protein can be recovered from the extract through selective ultra-filtration, the final product available commercially still consist of some impurities. Hence, the commercially available thaumatin sweetener is a mixture of thaumatins I and II along with other traces of the source material such as arabinogalactan and arabino glucuronoxylan polysaccharides (European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources Added to Food, 2015).

## BIOPRODUCTION

Due to the increasing awareness of the public over artificial sweeteners, natural sweeteners gained popularity. For instance, thaumatin is favored by the public to replace sucrose in food products. However, the fact that thaumatin is produced from a tropically grown plant limits its availability while the demand is high. Moreover, the production process can be greatly affected with respect to the source material availability and quality. In order to attain a more stable production of the protein to meet the demand, a series of studies has been performed involving its production through genetically engineered microorganisms and transgenic plants (Nabors, 2001; Jain and Grover, 2015; Masuda, 2016).

Commercially, proteins can be produced using techniques such as genetic and protein engineering. These proteins are used by the biopharmaceutical industry, enzyme industry and agricultural industry within the fields of medicine, diagnostics, food, nutrition, etc. In the early 1900s, the microbial fermentation industry came into the scene through the production of chemicals such as acetone, butanol, and citric acid. The first protein pharmaceutical that was produced through recombinant DNA technology and approved by the FDA was Human Insulin, in 1982. Some examples of other recombinant proteins are albumin, human growth hormone (HGH) and factor VIII. Furthermore, advancement in the technologies for production processes has contributed to the growth of the recombinant protein market. For example, the evolution in mammalian cell expression, baculovirus expression, *E. coli* expression and bioreactor systems have facilitated the ease of production of these proteins. Currently, more than 400 protein drugs achieved from recombinant technologies have been approved and marketed worldwide and more than 1300 of them are undergoing the process of attaining approval (Global Data, 2015)<sup>4</sup>. In 2016, the recombinant protein market witnessed a value of US\$ 347.2 million globally. According to information provided by the Coherent Market Insights company (2018), a compound annual growth rate of 6.2% is expected for the period 2017–2025<sup>5</sup>.

Numerous factors have to be considered for an efficient and low-cost production of recombinant proteins. Understanding the mechanism and metabolic pathway of thaumatin production can help in improving the yield and productivity. Since this protein has great potential as a substitute for the less safe sweeteners or sugar alternatives, an improved and commercially viable production is required. However, there is still much work to be done for the development of novel methods that can be utilized for the production of such recombinant proteins.

## Host Microorganisms

The two major factors that need to be taken into account for the expression of recombinant proteins are cloning of

<sup>4</sup>[www.globaldata.com/global-data-2015](http://www.globaldata.com/global-data-2015)

<sup>5</sup><https://www.coherentmarketinsights.com/market-insight/recombinant-protein-market-1516>

the desired DNA and the amplification of the protein in the chosen expression system. Selection of the expression system can be based on certain characteristics such as; quality of the protein, functionality, productivity, and yield (Demain and Vaishnav, 2009). Also, for the production of thaumatin, a suitable expression system needs to be chosen. Several systems are available for protein expression such as bacteria, yeasts, molds, mammals, plants or insects, transgenic plants, and animals. A wide number of studies has already been reported on the expression of thaumatin in microorganisms. Several of these studies reported that the protein yield was low and that the attained product was inactive (Edens et al., 1982; Edens and van der Wel, 1985; Illingworth et al., 1988, 1989; Lee et al., 1988; Hahm and Batt, 1990; Faus et al., 1997). In other cases, a higher yield could be attained through the use of artificial genes and codon optimization. For example, in Daniell et al. (2000) an acceptable yield could be achieved through the utilization of artificial genes with optimized codon usage encoding thaumatin II. This required a renaturation process because the recombinant proteins were attained as inclusion bodies which were insoluble and inactive. As such, the use of recombinant microorganisms is definitely not a straightforward route and considerable research has been undertaken for the purpose of optimizing the recombinant production of thaumatin.

An approach toward the usage of transgenic plants has also been tested in a number of studies for the synthesis of sweet proteins. The usage of plant cells or tissue culturing has certain advantages in various applications such as (i) post-translational modification similar to mammalian cells, (ii) no health issues on humans and (iii) simplified large-scale production (Doran, 2000; Hellwig et al., 2004). However, it has certain drawbacks when compared to the microbial production. For instance, the attempts on producing sweet-proteins especially, thaumatin in potatoes, strawberries, etc. showed a lower level of accumulation. A sufficient amount of the recombinant thaumatin is achievable from transgenic barley and tomato. However, there is significant scope for improvement regarding the extraction and purification of the protein (Firsov et al., 2016). Even though the usage of plant system has shown certain advantages over microbial systems in terms of scalability, economy and safety, they still lack some benefits attainable from the microbial hosts such as the possibility to control the growth conditions and product consistencies throughout the batches. Additionally, a further improvement on protein stability and recovery is required (Hellwig et al., 2004). Moreover, considering some of the potential issues associated with the use of transgenic plants, such as allergic reactions and regulatory uncertainties, limits it from a wider application. As such, the following sections are limited to the production of recombinant thaumatin using microorganisms as a host.

## Bacteria

*Escherichia coli* is one among the earliest and most commonly used host organism for protein expression (Terpe, 2006) due to the fast growth and expression, ease of culture and high yields (Swartz, 1996). Moreover, the genetics of *E. coli* are well-understood when compared to other organisms. Despite its great advantages, *E. coli* has certain drawbacks that can possibly

influence the production efficiency of recombinant proteins. This is because a high cell density of this organism results in large quantities of acetate, which is toxic to cells. It is also reported through studies that this organism fails to produce very large proteins. Additionally, some issues were observed for *E. coli* system in terms of difficulties in producing proteins that have disulfide bonds and refolding ability. Another factor is the failure to produce modified protein due to the absence of glycosylation (Jenkins and Curling, 1994). Glycosylation affects properties such as solubility, stability functionality, immunogenicity, etc. In order to achieve glycosylation for attaining a stable and properly folded protein, a higher advantage is noticed for recombinant production in yeast, mold, insect or mammalian cells. A solution can be opting for secretion of the heterologous protein into the medium instead of intracellular inclusion bodies. This way, soluble and active proteins can be attained with a much easier downstream processing and cost reduction (Mergulhão et al., 2005). Emergence of other bacterial systems can also be noticed over the years in the field of recombinant protein production. The engineering of *Lactococcus lactis*, a gram-positive bacterium for membrane protein expression is one such example (Chen, 2012). Additionally, they also possess advantages over *E. coli* in terms of being GRAS and endotoxin free (Yeh et al., 2009). *Pseudomonas* species such as *P. fluorescens*, *P. aeruginosa*, and *P. putida*, were also found as suitable alternatives to *E. coli* expression systems to attain higher yields of the recombinant protein. Today, several other bacterial systems are widely explored as cell factories. The authors Ferrer-Miralles and Villaverde (2013) have summarized in their paper the most important bacterial hosts that can be utilized as cell factories for recombinant protein production.

The thaumatin II gene has been cloned into *E. coli* K12 by Edens et al. (1982). However, this resulted in a very low production of the protein. Later, Faus et al. (1996) managed to express a synthetic gene encoding the same DNA sequences of thaumatin II successfully in *E. coli*. An immunoblotting analysis confirmed that the expressed thaumatin had a similar molecular weight as that of the plant source. Followed by this, an attempt by Daniell et al. (2000) replicating the same system resulted in approximately 40 mg of purified thaumatin. This product showcased a similar threshold value of sweetness as that from the natural origin.

## Fungi

A myriad of studies is available on bioproduction activities using *Aspergillus* and *Escherichia* species. However, the utilization of a yeast and other fungi species is found predominantly favorable due to the complexity in the growth of *Aspergillus* and lower yield in *Escherichia*. Considering the two most utilized organisms for the production of these relevant proteins; *Saccharomyces cerevisiae* and *Pichia pastoris*, both have the capability to produce proteins that are larger than 50 kDa in high yields and a possibility for glycosylation. Yeasts have the ability to produce chaperonins that can help in the folding of certain proteins and can handle S-S rich proteins (Demain and Vaishnav, 2009). With respect to thaumatin production, yields higher than 100 mg/L have been attained from yeast (Masuda and Kitabatake, 2006).

Gellissen et al. (2005a) emphasizes that yeast has more favorable conditions over other eukaryotic and prokaryotic systems to produce recombinant proteins thereby, resulting in yields of multigram range. The compact genome of yeasts makes the gene identification process much simpler (Goffeau et al., 1996). Moreover, they are robust, fast growing with a short lifecycle of 90 min and can be easily manipulated. Yeasts are easy to use for fermentations involving rapid growth into high cell densities on simple media. They also possess certain safety aspects by not involving with pathogens, viral inclusions, or pyrogens. Moreover, these eukaryotes have the ability to secrete and modify proteins (Gellissen et al., 2005b). It also comes with the advantage of DNA transformation facilitating gene cloning and genetic engineering. Thanks to all of these advantages, the application of yeast expanded widely from the chemical and enzyme production to the production of biopharmaceutical ingredients.

### *Saccharomyces cerevisiae*

One such yeast, *S. cerevisiae*, has been utilized as an ideal organism for several biological studies. For instance, the ethanol industry prefers this yeast for fermentation of raw materials such as sugar cane and beets or corn and wheat due to its high industrial potential. *S. cerevisiae*, commonly known as Baker's yeast, is one of the most industrially relevant eukaryotic microorganisms, mainly for bioproduction due to its availability, compatibility and more importantly, knowledge access of its genetic and physiological background. The genetic manipulation of this organism is comparatively easy and also widely accessible due to the available collections of genetic tools. Moreover, they exhibit a fast growth within a protein-free media, possess the capability for post-translational modifications and extracellular secretion (Tyo et al., 2014; Tang et al., 2015). Due to these properties of the organism, they have been widely used for bioprocesses and recombinant protein expression (Mattanovich et al., 2014). Such a platform is a prerequisite for the expression and production of heterologous proteins within the microbial system. However, while selecting an expression host it is important to note that overexpression of recombinant proteins can lead to intracellular accumulation and reduction of yields (Idiris et al., 2010; Tyo et al., 2014). It has to be taken into account that *S. cerevisiae* organism also possesses certain limitations. For example, Gellissen et al. (2005a) details that this microorganism has the tendency to hyperglycosylate recombinant proteins and results in batch inconsistencies due to strain instabilities caused by the use of episomal vectors.

The expression of preprothaumatin in *S. cerevisiae* was demonstrated by Edens et al. (1984) using a promoter fragment of the glyceraldehyde-3P-dehydrogenase (GAPDH) gene. Another yeast, *Kluyveromyces lactis* was also expressed with recombinant thaumatin II but a lower secretion of the protein was observed (Edens and van der Wel, 1985). An alternative solution to overcome these limitations can be by utilizing the non-conventional yeasts which have started gaining interest over the past decades (Reiser et al., 1990). This class of yeast includes mainly *P. pastoris* (Ilgen et al., 2005), *Hansenula polymorpha* (Gellissen, 2000, 2002; Kang and Gellissen, 2005), *Candida*

*boidinii* (Sakai et al., 1996), *Pichia methanolica* (Raymond et al., 1998), *Arxula adeninivorans* (Böer et al., 2005), *K. lactis* (Gellissen and Hollenberg, 1997), and *Yarrowia lipolytica* (Madzack et al., 2005). These methylotrophic yeasts have the ability to utilize methanol as a sole carbon source for carbon and energy. The growth associated with the utilization of methanol is possible due to the presence of alcohol oxidase that is not included in the glucose-grown cells. Out of these, *H. polymorpha* and *P. pastoris* have been extensively used as academic tools and for production of proteins that are commercially available today.

### *Pichia pastoris*

Given the specific successes that have been obtained with the production of recombinant thaumatin in *P. pastoris*, this microorganism is discussed here in more detail. Similar to *S. cerevisiae*, *P. pastoris* also has a pertinent fermentative growth. *P. pastoris* was first isolated from the chestnut tree in 1919 and was described as *Zygosaccharomyces pastori* (Guilliermond, 1920). However, in the 1950s different strains related to this yeast were isolated from Oak trees by Herman Phaff and he renamed it as *P. pastoris* (Phaff et al., 1956). Close to 10 years back, *P. pastoris* was re-classified into the genus *Komagataella* and since then given the name *Komagataella phaffii*. The protein production platforms utilized today are technically speaking either *K. phaffii* or *K. pastoris*, which are the same (Kurtzman, 2009).

A further classification can be considered for the *P. pastoris* strains. This can be wild-type strains (X-33, Y-11430), auxotrophic mutants that are defective in histidinol dehydrogenase (e.g., GS115), mutants that are defective in genes involved in methanol utilization (KM71, MC 100-3) and protease-deficient strains (SMD1163, SMD1165, SMD1168) (Jahic et al., 2006). *P. pastoris* is a yeast belonging to an integral part within the field of bioproduction for the past years and is foreseen to remain significant also in the future. It is a widely used single-celled organism for recombinant protein production due to the advantages it exhibits compared to other microorganisms. Moreover, it is a Crabtree-negative yeast meaning that it has preference for respiration rather than fermentation. Unlike Crabtree-positive yeasts, they do not use up the carbon source by producing ethanol and instead facilitate the production of higher biomass constituting toward more recombinant production. This makes *P. pastoris* more suitable for recombinant protein production than Crabtree-positive yeasts such as *S. cerevisiae*.

All strains of *P. pastoris* are derived from the wild-type NRRL Y-11430 (Northern Regional Research Laboratories) (Macauley-Patrick et al., 2005). In addition to sharing a similar intracellular environment and post-translational protein processing capacities with higher eukaryotes, the proteins generated from these strains are presumably correctly processed. Expression of recombinant proteins in the methylotrophic *P. pastoris* offers the possibility for an easier and faster production of high quantities of the protein. Usage of such a system facilitates the construction of heterologous protein in a more straightforward manner. This can be mainly attributed to the ease of genetic manipulation of this species and its fast growth on inexpensive media resulting in high cell densities. Moreover, this yeast has the capability to perform post-translational modifications including protein



folding, proteolytic processing, disulfide bond formation and glycosylation (Cereghino et al., 2002). Additionally, it comes with the advantage of simple a purification process due to the low levels of native proteins that gets secreted (Cregg et al., 1993).

*Pichia pastoris* also has the capability to produce from milligram-to-gram quantities of proteins, making it suitable for both laboratory research and industrial manufacturing (Hellwig et al., 2001). Hence, this yeast has been utilized as an ideal host for higher production of different recombinant proteins (Tuite et al., 1999; Cregg et al., 2000; Cereghino and Cregg, 2000; Cereghino et al., 2002; Macauley-Patrick et al., 2005). From past literature, it can be observed that this strain has been successfully utilized as a host for more than 600 recombinant proteins (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005). From the discussion provided by the various authors it can be understood that *P. pastoris* plays a significant role in the production of recombinant proteins (Cregg et al., 2000; Cereghino and Cregg, 2000; Abad et al., 2010), especially for the production of complex proteins which need post-translational modifications and those with disulfide bridges (Cereghino et al., 2002). Therefore, more attention will be given to the bioproduction of thaumatin with *P. pastoris* in the remainder of this review.

### Filamentous fungi

Filamentous fungi have placed a mark in industrial bioproduction due to their ability to produce primary metabolites such as organic acids, human therapeutics, fungal enzymes, and single cell protein. As such, the production of recombinant proteins is not just limited to bacteria and yeasts. Filamentous fungi have the capability to grow at high rates and densities in the presence of inexpensive media using simple fermenters. *Aspergillus awamori* was also utilized for attaining a higher yield of recombinant thaumatin which resulted in a non-homogeneous product consisting of three forms of thaumatin (Moralejo et al., 1999, 2001; Lombrana et al., 2004). The yield of this production was 5–7 mg/L as seen in **Table 4**. It can be noticed from these studies that *A. awamori* performed well-compared to the other organisms.

Filamentous fungi have been utilized as an expression platform for screening and production purposes. These fungal-based systems exhibit certain benefits for the high-level secretion of enzymes and the large-scale production of recombinant proteins. However, compared to the bacterial system, filamentous fungi are more complex to understand in terms of their physiology, thereby hindering their potential as efficient factories for heterologous protein production (Su et al., 2012). Moreover, some strains can also be pathogens for humans, animals and plants (Maor and Shirasu, 2005; Cutler et al., 2006; Segal and Walsh, 2006).

### Upstream Processing

During a typical *P. pastoris* fermentation to produce heterologous protein, the strain is subjected to a variety of conditions that need to be selected, such as glycerol concentration and feeding for biomass accumulation, methanol feeding for induction of expression, temperature, pH, agitation rate, and dissolved oxygen within the bioreactor. It is vital to understand the influence of the

carbon source, promoter and temperature on the metabolism of *P. pastoris*. This information is crucial to avoid the activation of certain metabolic pathways and the production of enzymes that could affect the growth of the organism and recombinant protein production. During *P. pastoris* fermentation, a defined medium containing glycerol as the sole carbon source is preferred for the growth of the strains. After the accumulation of biomass, the production of the protein can be achieved by an induction stage through the addition of methanol.

The whole fermentation process can be explained in three main phases, i.e., glycerol batch phase, glycerol fed-batch phase, and methanol fed-batch phase. Firstly, the strain is grown within a defined medium which consist of glycerol as the sole carbon source. During this period the biomass accumulation takes place, but the heterologous gene is completely repressed. This stage is continued until a high cell density of the desired organism is attained and the carbon source gets depleted. At this point, the heterologous gene expression is repressed. An additional amount of glycerol is still fed into the reactor to facilitate the derepression of the cells and accumulation of biomass (fed-batch phase). This stage will be then followed by the fed-batch phase (transition or induction/production phase) which is initiated by the addition of glycerol-methanol mixture and later by pure methanol (D'Anjou and Daugulis, 2000). During this stage the gene expression takes place and the protein of interest is produced. The conditions inside the reactor can influence the yield of the protein produced and hence needs to be optimized in a way that leads to high cell densities and protein yields. The conditions for optimization in the reactor can be for instance medium composition, pH, temperature, and feeding profiles.

The first cloning of the natural thaumatin II cDNA was performed on the yeast-shuttle vector pPIC9K. This vector has an inducible alcohol oxidase 1 (AOX1) promoter, *S. cerevisiae* prepro  $\alpha$ -mating factor secretion signal and a kanamycin resistance gene which can be used for G418 selection (Scorer et al., 1994). Following this, the natural thaumatin II containing vector was transformed into *P. pastoris* GS115 from which approximately 25 mg/l recombinant thaumatin II was obtained. It was observed from further analysis that the obtained recombinant thaumatin contained extra amino acid residues at both ends, i.e., the N- and C- termini. Irrespective of the extra amino acid residues, the resultant recombinant thaumatin still elicited a similar sweetness to the one derived from natural source, demonstrating that the terminal regions do not interfere in the sweetness of the protein (Masuda et al., 2004). *P. pastoris* is seen as an ideal organism for the large-scale production of the recombinant proteins because the medium required for its growth is relatively simple constituting mainly glycerol and methanol as the main carbon sources and other components such as biotin, trace elements and salts. Moreover, they do not involve any components that can contribute to the production of any toxins, hence making it suitable for producing human pharmaceuticals as well. Additionally, the culture medium has less chance to get contaminated by other organisms since *P. pastoris* utilizes a low pH and methanol concentration. Thaumatin cDNA along with the  $\alpha$ -factor signal

sequence and the Kex2 protease cleavage site were introduced into *P. pastoris* in the study of Ide et al. (2007a). The analysis revealed that the N-terminal consist of two or three unexpected amino acid residues which might be due to the Kex2 protease deficiency resulting in the cleavage of the peptide bond. From the analysis of the N-terminal, it was noticed that some of the unexpected amino acids were attached to it. These amino acids do not affect the threshold value of sweetness of thaumatin (Ide et al., 2007a) but could possibly influence the expression, secretion and structure. The most commonly used *P. pastoris* strains used for the recombinant protein production are mentioned in Alkhalfioui et al. (2011).

The most commonly used *P. pastoris* strain for recombinant protein production is GS115, which is obtained from the wild-type strain NRRL-Y 11430 and has a mutation in the histidinol dehydrogenase gene (HIS4) (Schutter et al., 2009). *S. cerevisiae* and *P. pastoris* hold comparatively similar metabolism with respect to the regulation of the central carbon metabolism and flux ratio profiles for amino acid biosynthesis (Sola et al., 2004). The former has been extensively used for heterologous protein production in the past. Hence, this has the advantage of providing a large amount of data that can be utilized for the optimization of *P. pastoris* culturing in terms of growth and heterologous production. For instance, the minimal media required for the growth of *S. cerevisiae* can be optimized for the biomass production of *P. pastoris*. However, Papanikou and Glick (2009) mention in their study that *P. pastoris* and *S. cerevisiae* do not share the same morphology based on the differences in the Golgi apparatus which could be a reason for *P. pastoris* to secrete the recombinant proteins more efficiently.

Heterologous protein production can be achieved either intracellularly or secreted into the medium by utilizing a secretion signal sequence. The secreted production can be used to avoid problems related to the folding of the proteins and can simplify the purification process compared to the intracellular production. A widely used secretion sequence is the prepro-sequence of the alpha-factor structural gene (MF $\alpha$ 1) which is functional in all yeast strains (Brake et al., 1984). The advantage with the extracellular production from *P. pastoris* is that it secretes limited amounts of endogenous proteins and the culture medium does not contain any added proteins. Hence, the largest fraction of the obtained proteins in the medium will be thaumatin (Tschopp et al., 1987b; Bar et al., 1992).

**Table 5** showcases the use of different strains of *P. pastoris* for the production of thaumatin utilizing specific plasmids. Masuda

et al. (2004) was the first research to report the expression of thaumatin by a *P. pastoris* strain. Natural mature thaumatin II was produced at a yield of about 25 mg/L and was found to elicit a sweet taste, comparable to that of thaumatin from the natural source. Four years later, the thaumatin I variant was produced in *P. pastoris* by Ide et al. (2007a). The authors also attempted to determine common features between proteins eliciting a sweet taste but were unsuccessful. At the same time, Ide et al. (2007b) determined that the pre-sequence has an advantage for the secretion of thaumatin into the culture medium. This sequence is identical to the natural secretion signal. In Ohta et al. (2008), thaumatin mutants were produced to determine the amino acid residues that deliver the sweet taste. Two key amino acid residues (R82 and K67) were identified that played a key role in binding to the taste receptors. A further study on the intensely sweet taste of thaumatin II was made by Masuda et al. (2014). Masuda et al. (2010) used an expression vector that contained three copies of the thaumatin I gene to drive up the yield to 100 mg/L. Healey et al. (2017) tested the co-expression of thaumatin with disulfide isomerase to facilitate protein folding. This led to a doubling in the production rate of thaumatin. This evolution shows the large advances that have been made in understanding the sweetness and microbial production of thaumatin.

Alcohol oxidase (AOX) is the enzyme that catalyzes the first step in the methanol utilization pathway (Hartner and Glieder, 2006). In this step, methanol is oxidized to formaldehyde while reducing O<sub>2</sub> and H<sub>2</sub>O. Following this step, the formaldehyde either gets oxidized to CO<sub>2</sub>, thereby giving rise to two molecules of NADH, or gets condensed with xylulose 5-phosphate and then is converted to dihydroxyacetone and glyceraldehyde 3-phosphate within the methanol utilization pathway (Hartner and Glieder, 2006). *P. pastoris* contains two genes AOX1 and AOX2 that encodes two enzymes with AOX activity (Cereghino and Cregg, 2000). The former is controlled by the strong pAOX1 promoter and the latter by the weaker pAOX2 promoter. The AOX1 gene constitutes for most of the alcohol oxidase activity within the cell (Ellis et al., 1985; Tschopp et al., 1987a; Cregg et al., 1989). *P. pastoris* consists of three phenotypes which are involved with the methanol utilization. These include (i) methanol utilization plus (Mut<sup>+</sup>), where both genes are active and intact, (ii) methanol utilization slow (Mut<sup>s</sup>) where AOX1 is knocked out and (iii) methanol utilization minus (Mut<sup>-</sup>), where both AOX genes are knocked out when methanol is used as the sole carbon source (Cereghino and Cregg, 2000).

**TABLE 5** | Recombinant thaumatin production utilizing various strains of *Pichia pastoris*.

Thaumatin form	<i>Pichia</i> strain	Gene	Yield	Literature
Thaumatin II	GS115	Natural mature	25 mg/L	Masuda et al., 2004
Thaumatin I	X-33	Natural mature	30 mg/L	Ide et al., 2007a
Thaumatin I	X-33	Natural pre/pro	60 mg/L	Ide et al., 2007b
Thaumatin I	X-33	Natural pre	15 mg/L	Ohta et al., 2008
Thaumatin I	X-33/SMD1168H	Multiple copies	100 mg/L	Masuda et al., 2010
Thaumatin II	X-33	Natural pre	30–50 mg/L	Masuda et al., 2014
Thaumatin I	PPS-9010	Natural mature + chaperone	50 mg/L	Healey et al., 2017

In the Mut<sup>-</sup> strain methanol acts more like an inducing agent for the production of the recombinant proteins since they are unable to metabolize it Chiruvolu et al. (1997). The Mut<sup>+</sup> strains showcased a maximum specific growth rate of 0.15 h<sup>-1</sup> at an excess concentration of methanol as substrate and a temperature of 30°C (Kobayashi et al., 2000).

## Downstream Processing

Purification and recovery of recombinant proteins is a crucial step in a bioprocess. This may include a series of techniques depending on the protein of interest and the mode of production. In the case of downstream processing following an intracellular production, the washed and resuspended cell pellets are broken by vortexing with glass beads and the supernatant containing the cytosolic proteins is recovered by centrifugation. The concentration of the proteins could be determined through the bicinchoninic acid assay (Ide et al., 2007a). One of the major limitations associated with the recovery of heterologous proteins from the *P. pastoris* system would be proteolysis of the secreted proteins and cell death within the bioreactor. This can result in lower quality of the product after the downstream processing (Jahic et al., 2006).

A simplified purification process can be applied when using secreted production. This secreted production is often possible for foreign proteins that are secreted naturally by their native hosts. This comes with the requirement for a signal sequence to guide the protein through the secretory pathway. The native secretion signal sequences have been used extensively. However, the most successful has been identified as the *S. cerevisiae*  $\alpha$ -factor prepro-peptide. This signal sequence includes a 19-amino acid signal (pre) sequence and a 66-residue (pro) sequence consisting of *N*-linked glycosylation sites and a Kex2 endopeptidase processing site (Kurjan and Herskowitz, 1982). Brake et al. (1984) elaborates on the three major steps involved in processing the signal sequence. If the protein secretion is successful, the next important step is to recover the protein and attain it in purest form. The recovery of the protein of interest in the most economical way requires an advanced downstream processing and purification. Proteins attained through secreted production facilitate an easier recovery through filtration-based purification and concentration.

Several papers discuss on the possible analytical methods that can accommodate the characterization of thaumatin. For instance, Masuda (2016) elaborates in his paper on the use of an SP-Sephadex C-25 column to separate the dialyzed culture supernatant. Followed by this step, the desired fraction was precipitated using ammonium sulfate. It is further dialyzed and applied to a Toyopearl HW-50F column and eluted with a buffer. The attained protein is checked for its purity by utilizing the SDS-PAGE and native PAGE techniques and quantified using the bicinchoninic acid method. The purification of the protein can depend on the type of protein and tag used.

One of the common methods adopted for the purification of recombinant proteins is through chromatography, specifically affinity chromatography because of their high specificity. Tags are used for improving the solubility and for facilitating affinity

purification. These tags are small structures with short sequences of just 3–4 amino acids that can be added to the recombinant protein and hence allows to capture the proteins with ease. Apart from these, some fusion tags can be useful for a range of applications such as labeling for imaging studies, localization studies, detection, quantification, protein–protein interaction studies, subcellular localization or transduction and many others (Malhotra, 2009). Hence, the selection of an appropriate tag while designing the expression constructs is an important step and often puts the researcher in a dilemma to choose. The common tags associated with the recombinant protein production are histidine tag, glutathione-S-transferase, maltose binding protein, etc. The tags can be positioned at the N- or C- terminus of the protein of interest. However, placing them at the N-terminal comes with the advantage of solubilizing the target proteins (Sachdev and Chirgwin, 1998) and the easy removal of the tag. Since these tags have an influence on the structure and function of the end protein, it is necessary to remove them during the purification step with the usage of endoproteases such as tobacco etch virus protease or thrombin.

## PERSPECTIVES

Several biopharmaceuticals are nowadays produced by the utilization of technologically advanced microbial and mammalian cell biosystems. This technique provides advantages for the production of important recombinant pharmaceuticals in a safe and abundant quantity. The utilization of a high number of engineered strains of *P. pastoris* and the designing of metabolic pathways for the enhanced production of the heterologous proteins have been seen in recent years. Today, *P. pastoris* is extensively utilized as a successful expression system for both industrial and research purposes (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005). The use of recombinant DNA helps in the amplification and cloning of important genes within *P. pastoris*. As such, there is a plethora of opportunities for the production of protein of interest with this emerging technique. The metabolism includes several coupled and interconnecting reactions. The term metabolism refers to a reaction comprising the conversion of a molecule into another molecule or molecules within a defined pathway. A cell includes numerous pathways of such reactions which are independent and that can be coordinated with the presence of enzymes. Understanding the metabolic reactions gives insights on the information related to the fluxes. This information leads a path for the establishment of the metabolic model which can serve as a tool for setting up an optimized bioreactor process. Such a tool can be advantageous to predict the parameters influencing the process or genetic modifications on strain behavior (Kerkhoven et al., 2014; Cankorur-Cetinkaya et al., 2017).

Considering the increased interest in *P. pastoris*, methods for increasing the production of recombinant proteins with this host are of high relevance (Zhang et al., 2005). This can be achieved by genetically engineering the metabolism of the microorganism in such a way that the product yield is maximized. In general, the processes involved in a microbial metabolism are complex and a

significant effort is required to study these processes. Specifically, for *P. pastoris*, there are still some limitations persisting for the engineering of this non-conventional yeast, due to a lack of genome editing tools and incomplete knowledge on their cell behavior when compared with the yeast platforms such as *S. cerevisiae*. As such, this line of work comes with the limitations of time consumption, high cost and production of noisy data. Therefore, another method to be adopted can be the development of mathematical models which have the ability to characterize the various phenotypes their culturing behavior (Sidoli et al., 2004; Royle et al., 2013).

Even though genetic engineering is a valuable first step, on a large-scale production of these compounds, a further optimization of the process based on carbon and nitrogen sources, feeding profiles, aeration levels and so on will be necessary as well. In order to perform this optimization in a systematic manner, it is necessary to learn how the behavior of *P. pastoris* during culturing can be predicted. Therefore, the key factors influencing microbial growth and protein production should be determined and the relationships between them should be captured in a mathematical model. An appropriate model structure should describe effects that limit recombinant protein production such as cell viability, metabolite accumulation, metabolite toxicity, substrate requirement, and substrate inhibition.

## Systems Biology

Systems biology is an approach which helps in studying the interactions within biological systems. It facilitates in understanding the behavior of systems involving several enzymes, metabolites and metabolic pathways or signaling networks. This understanding is obtained with the aid of mathematical models and computational techniques (Vidal, 2009). Hence, it can be deduced from available literature that systems biology adopts a holistic approach involving a complete system in comparison to molecular biology that involves subsystems through *in vitro* studies (Kell and Oliver, 2003). This approach relies on the vast amount of biological information that is available on several biological systems. However, the integration of significant amounts of high-quality data is often still needed to obtain the desired insights.

The utilization of microbial cell factories within industrial microbiology involves a constant process of strain engineering and optimization of the bioprocessing conditions. The main target with respect to the production of thaumatin would be to accommodate the maximum microbial production rate. For this, an optimal environment needs to be provided toward the culturing of the microorganism on one hand and the production of thaumatin on the other. With this respect, the media composition has a major influence on the overall performance of the bioprocess. Systems biology provides highly effective tools such as flux balance analysis and metabolic flux analysis for the optimization of media composition. However, both require that information on the metabolic network of the microorganism and the latter also requires a significant amount of experimental data. There has been sufficient data available already from studies depicting that an optimized nutritional

medium is required for the organism to enhance the recombinant protein production. However, in order to obtain a deeper knowledge on the actual effect of media composition on cell growth and product formation, additional data is still required. The data itself can be from cell physiology (Rehberg et al., 2013), extracellular metabolites (Genzel et al., 2004), intracellular metabolites (Ritter et al., 2006), and enzyme activities (Janke et al., 2010). Most importantly, this data should provide information on the metabolic response of the selected organism. This response can be quantified using mathematical modeling techniques.

The inclusion of microscopic models has the advantage of resulting in more mechanistic and generic predictive models when compared to the macroscopic models that do not take into account the underlying mechanisms that cause a certain response. For example, macroscopic models will simply describe the production rate of the end-product based on some key influencing factors whereas microscopic models will consider in more detail the metabolic reactions that lead to the production of this product. Despite the fact that the metabolism of, e.g., *P. pastoris*, has been studied over the years, many parts of it still remain unclear. For instance, the secondary metabolism is very diverse in addition to the complexity existing between the connections in the various reactions thereby resulting in an extremely dense biological network. The *in silico* reconstructions of the metabolism can be achieved from the genome-scale metabolic models. Such a model helps in the linkage between the genotype and metabolic capability.

## Genome-Scale Metabolic Models

When looking into the traditional techniques used for modeling bioreactor processes, they are derived from mass balances of biomass and growth limiting carbon source. Similar model types can be utilized for the bioproduction of *P. pastoris*. These classical approaches are based on a system with ordinary differential equations or their explicit form. However, this information alone does not provide mechanistic knowledge on the cell. Further advancements have taken place with respect to model development through the integration of information on the metabolic level. These types of studies have been performed as well for *P. pastoris* strains. As such, the emergence of the implementation of genome-scale metabolic models has magnified the knowledge on the cell behavior. The first such model constructed for a eukaryotic organism was for *S. cerevisiae*. Now, there have been many studies involved in the reconstruction and expansion of the metabolic networks

**TABLE 6** | Available genome-scale metabolic models for *P. pastoris*.

Model	Reactions	Metabolites	Reference
iPP668	1354	1177	Chung et al., 2010
PpaMBEL1254	1254	1058	Sohn et al., 2010
iLC915	1423	1302	Irani et al., 2015
iMT1026	2035	1689	Tomàs-Gamisans et al., 2016
iRY1243	2407	1740	Ye et al., 2017
iFS670	1383	1195	Saitua et al., 2017



for yeasts. The various reconstructions of the genome-scale metabolic network models for *P. pastoris* are given in **Table 6**.

The use of metabolic models helps in improving the production of target compounds and for uncovering new information on the biological systems. Hence, these models play a significant role in the design and optimization of bioprocesses. This way, a more quantitative and systematic framework that has the potential to improve the value and reliability can be achieved. It is noticed from recent literature that the engineering approach toward cell metabolism boosts the productivity of recombinant proteins. This is evident from the high number of pathway designs and engineered constructs that are available for *P. pastoris* today. A full metabolism involves numerous reactions and hence, utilizing mathematical models contributes toward a better understanding of this complex process.

The optimization of fermentation processes requires the selection of numerous conditions such as pH, temperature, oxygen concentration and nutrient supply (Cos et al., 2006; Jahic et al., 2006; Calik et al., 2015). The use of a mathematical model is significant for the conversion of the available experimental data into new insight (Nielsen, 2017) and helps in getting information in a concise manner. However, several aspects on the true mechanics of the bioprocess still remain unknown, limiting the possibility to build a model that depicts the full set of cellular processes for a given microorganism. Most of the studies related to the metabolic modeling of *P. pastoris* are based rather on the influence of protein production on the metabolism than the prediction on specific efficiency. Hence, the genome-scale metabolic models seem to be a very valuable tool for the optimization of biochemical production processes that are still not used to their full potential.

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## CONCLUSION

Sweet proteins are seen to have great potential today in various applications and are expected to rise to a level capable enough to replace sucrose in several applications. Due to their low-caloric feature and great qualities as a sugar replacer, the consumers have perceived these natural sweeteners with a positive outlook. Thaumatin specifically is emerging as a strong alternative to sucrose and other synthetic replacers in the market today. Although thaumatin has been studied by various researchers over the past 30 years, there is still much work to be done in order to improve its production via the biochemical routes. It is evident from the literature that biological products are emerging as a promising applicant within the food industry. Hence, there is great potential for future research that focuses on the use of advanced computational techniques for the optimization of thaumatin bioproduction.

## AUTHOR CONTRIBUTIONS

JJ delivered the main contribution in reviewing literature and writing, in close collaboration with SA and PN. JI supervised the writing of this review. The general content and lay-out of this publication were determined by all authors.

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# Use of Mass Spectrometry to Profile Peptides in Whey Protein Isolate Medium Fermented by *Lactobacillus helveticus* LH-2 and *Lactobacillus acidophilus* La-5

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Peptides in the 3-kDa ultrafiltrate of fermented whey protein isolate (WPI) medium could be responsible for the antivirulence activity of *Lactobacillus helveticus* LH-2 and *Lactobacillus acidophilus* La-5 against *Salmonella* Typhimurium. Non-fermented and fermented media containing 5.6% WPI were fractionated at a 3 kDa cut-off and the filtrate was analyzed by mass spectrometry. The non-fermented WPI medium contained 109 milk derived peptides, which originated from  $\beta$ -casein (52),  $\alpha$ s1-casein (22),  $\alpha$ s2-casein (10),  $\kappa$ -casein (8), and  $\beta$ -lactoglobulin (17). Most of these peptides were not found in the fermented media, except for 14 peptides from  $\beta$ -casein and one peptide from  $\alpha$ s2-casein. Database searches confirmed that 39 out of the 109 peptides had established physiological functions, including angiotensin-converting-enzyme (ACE) inhibitory, antioxidant, antimicrobial, or immunomodulating activity. A total of 75 peptides were found in the LH-2 cell free spent medium (CFSM): 54 from  $\beta$ -casein, 14 from  $\kappa$ -casein, 4 from  $\beta$ -lactoglobulin and 3 from  $\alpha$ s2-casein. From these peptides, 19 have previously been associated with several categories of bioactivity. For La-5 CFSM, a total of 15 peptides were sequenced: 8 from  $\beta$ -casein, 5 from  $\alpha$ s1-casein, 2 from  $\beta$ -lactoglobulin. Only 5 of these have previously been reported as having bioactivity. Many of the peptides remaining in the fermented medium would contain low-affinity residues for oligopeptide binding proteins and higher resistance to peptidase hydrolysis. These properties of the sequenced peptides could explain their accumulation after fermentation despite the active proteolytic enzymes of LH-2 and La-5 strains. Down-regulated expression of *hilA* and *ssrB* genes in *S. Typhimurium* was observed in the presence of La-5 and LH-2 CFSM. Downregulation was not observed for the *Salmonella oppA* mutant strain exposed to the same CFSM used to treat the *S. Typhimurium* DT104 wild-type strain. This result suggests the importance of peptide transport by *S. Typhimurium* for down regulation of virulence genes in *Salmonella*.

**Keywords:** bioactive peptides, probiotic, whey, *Salmonella*, virulence, gene expression

## INTRODUCTION

*Salmonella enterica* subsp. *enterica* serovar Typhimurium is considered a major foodborne pathogen with public health and economic concerns. This foodborne pathogen has developed resistance against a broad range of antibiotics (1). Alternative approaches to control this pathogen depending on the inhibition of virulence gene expression and stimulation of the host immune system have been suggested. These approaches cause less stress to bacterial cells to avoid the development of resistant clone (2). A Type III Secretion System (TTSS) is used by *S. Typhimurium* to inject and translocate effector proteins into host cells for adherence, attachment and invasion (3). *Salmonella* pathogenicity island I (SPI1) controls the production and activity of the Type III secretion system which enables the membrane ruffling process, in which the epithelial cell cytoskeleton is rearranged to engulf the *Salmonella* into cytoplasmic vacuoles (4). The gene *hilA* directly controls and activates all the genes of SPI1 for invasion (5). The gene *ssrB* is the main regulator of SPI2, which is responsible for systemic infection and replication of *S. Typhimurium* inside macrophages and epithelial cells (6). Down regulation of these virulence genes would be an alternative way to reduce the severity of *Salmonella* infection.

Milk proteins are a major source of bioactive peptides (7). Milk derived peptides may have effects on the digestive, immune, cardiovascular and nervous systems (8). Previous studies have suggested that bioactive metabolites produced by *L. helveticus* LH-2 and *L. acidophilus* La-5 could down-regulate virulence gene expression in both *Salmonella* and *E. coli* O157:H7 after growth in chemically defined media and milk (9–12).

Bioactive peptide production in lactic acid bacteria (LAB) is the result of the balance between proteolytic activity and peptide consumption (13). The proteolytic system of LAB consists of proteinases and peptidases which initially cleave milk proteins to oligopeptides, specific transport proteins which transport smaller peptides and amino acids across the cytoplasmic membrane and intracellular peptidases, which further degrade these peptides to small peptides and amino acids (14). The considerable amino acid auxotrophy of *L. helveticus* and *L. acidophilus* is consistent with the presence of many peptidases/proteases and related transport systems for amino acids and peptides (15).

The oligopeptide-binding protein (OppA) is one of the most abundant periplasmic proteins in *S. Typhimurium*. *oppA* gene expression in *S. Typhimurium* is modulated by nitrogen compounds and regulates the expression of other genes such as Lrp (leucine-responsive regulatory protein) or CodY (16). Mutation in OppA in *S. Typhimurium* leads to losing the ability to transport muropeptides and detect the nutritional or signaling peptides (17).

In this study, the proteolytic and peptidolytic activities of LH-2 and La-5 strains in Whey Protein Isolate (WPI) are explored through genome sequencing and biochemical assays on substrates. Peptide profile analysis by mass spectrometry was conducted to understand the potential factors that lead to accumulation of antivirulence peptides in the growth media. Furthermore, the correlation between *oppA* gene expression and virulence gene expression in *Salmonella* in the presence of

antivirulence peptides was investigated using a *S. Typhimurium oppA* mutant strain.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

All bacterial strains used in this study were obtained from the Canadian Research Institute for Food Safety (CRIFS) except for the *Salmonella Typhimurium oppA* mutant, which was provided by Prof. Eric Brown, McMaster University. *Lactobacillus helveticus* LH-2 and *Lactobacillus acidophilus* La-5 probiotic strains were grown under anaerobic conditions at 37°C for 48 h on De Man, Rogosa, Sharpe broth (MRS; Thermo Scientific™ Oxoid™ Ottawa, ON, Canada). *Salmonella Typhimurium hilA::lux* and *Salmonella Typhimurium ssrB::lux* were grown on Luria-Bertani broth (LB; Life Technologies, Burlington, ON, Canada) supplemented with 50 µg/ml of ampicillin (Amp). Both constructs were grown aerobically overnight on a shaking incubator at 37°C. *S. Typhimurium* DT104 and *S. Typhimurium oppA* mutant strains were grown under the same condition as *S. Typhimurium* constructs, but without Amp. Solid media preparation for all strains were carried out under the same conditions but with addition of 15 g/l of agar and incubated under the same conditions.

### Preparation of *L. helveticus* LH-2 and *L. acidophilus* La-5 Cell Free Spent Medium (CFSM)

Whey Protein Isolate (WPI) (NZWPI 895, Caldic, Fonterra, USA) was dissolved at 5.6% (v/v) in sterile sugar solution. Glucose or sucrose (Fisher Scientific, Ottawa, ON, Canada) at 0.5% (v/v) were added for *L. helveticus* LH-2 and *L. acidophilus* La-5, respectively. The media were filter sterilized through 0.45 µm pore-size filters (Corning, NY 14831, Germany). An overnight culture of each strain in MRS broth was washed and inoculated at 5% (v/v) into WPI based media and incubated anaerobically at 37°C for 48 h. Following growth, bacterial cells were removed by centrifugation at 15,000 × g for 30 min at 4°C. The supernatant was filtered through a 0.20 µm pore-size filter (Corning) to obtain sterile CFSM which was subsequently freeze-dried (VirTis, Genesis, USA) for 72 h and stored at –80°C.

### Effect of CFSM on Gene Expression in *S. typhimurium* Reporter Constructs

For initial screening of the antivirulence effects of CFSM, bioluminescent reporter strains *S. Typhimurium hilA::lux* and *ssrB::lux* (18) were used. Briefly, the *luxCDABE* operon from *Xenorhabdus luminescens* was isolated and cloned with an Amp resistance gene into a plasmid (pSB377), which was further fused with the *hilA* and *ssrB* promoter regions, separately. The expression of the *lux* genes is controlled by these promoter regions, so that light is emitted when the gene is expressed. Each overnight culture of the constructs was diluted 1:100 with fresh LB broth with and without supplementation of 10% (v/v) of neutralized (pH 7) CFSM from *L. helveticus* LH-2 and *L. acidophilus* La-5, which had been reconstituted in



sterile deionized water at 10-fold concentration compared to the initial medium. The samples were incubated aerobically at 37°C with shaking for 3 h. Luminescence was measured with the FB 12 luminometer (Berthold Detection Systems, Pforzheim, Germany). The results are presented as Relative Light Units (RLU)/OD<sub>600</sub> nm.

## Effect of CFSM and Mixture of Synthetic Peptides on Transcription of Virulence Genes of *S. typhimurium* by RT-qPCR

### RNA Extraction

RNA was extracted from *S. Typhimurium* DT104 and *S. Typhimurium oppA* mutant following 3 h growth in fresh LB broth with and without 10% neutralized (pH 7) CFSM with shaking at 37°C, 2-ml samples were centrifugated at 5,000 × g for 4 min at RT. The supernatant was discarded and cells were mixed with 1 ml of RNA Protect reagent (Qiagen Inc., Mississauga, ON, Canada) and incubated for 5 min at RT. Cells were collected again by centrifugation at 5,000 × g for 10 min at RT. Cell pellets were suspended in 200 µl of Tris-EDTA buffer, pH 8.0 (Fisher Scientific, Ottawa, ON, Canada), 60 µl of 20 mg/ml lysozyme (Fisher Scientific) and 20 µl of proteinase K (Qiagen). The suspensions were incubated at 37°C for 1 h with shaking at 450 rpm. RNeasy Plus Mini Kit (Qiagen) was used to extract RNA from all samples following the manufacturer's instructions. DNA was eliminated by using RNase-Free DNase Set (Qiagen). In brief, 40 µl of total RNA was incubated for 10 min at RT with 2.5 µl DNase I stock solution, 10 µl RDD buffer in a total volume of 100 µl. RNA purification and concentration were performed with RNeasy MinElute Cleanup kit (Qiagen) and solubilized in 30 µl molecular-grade water. The quantity of quality RNA was determined by measuring the absorbance at 260 and 280 nm using a NanoDrop 1000 spectrophotometer (Thermoscientific, Wilmington, DE 19810, USA). RNA integrity was verified by gel electrophoresis.

The same procedures were used for a mixture of the following synthetic peptides (GLDIQKVAGT, ELNVPGEIVES, DVENLHLPLPL, GVSKEAMAPKH, SSSEESITRIN) (Synpeptide) at a concentration of 0.2 mg/ml for each peptide.

### Reverse Transcription

The purified RNA was used for reverse transcription by using high-capacity cDNA reverse transcription kit (Applied Biosystems, Burlington, ON, Canada). RNA (1 µg) was mixed with 2 µl of 10× RT buffer, 2 µl of 10× random hexamer primers, 0.8 µl of 25× dNTP (100 mM), 1 µl of Multiscribe reverse transcriptase (50 U/ml) in a total volume of 20 µl.

A no reverse transcription control was included to confirm the absence of contaminating DNA. The synthesis of cDNA was conducted using a Mastercycler Gradient Thermocycler (Eppendorf, Mississauga, ON, Canada) under the following settings: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and a holding step at 4°C. The cDNA was stored at -20°C until use.

### RT-qPCR

A ViiA<sup>TM</sup> 7 Real-Time PCR System (Applied Biosystems, Burlington, ON, Canada) and PowerUp<sup>TM</sup> SYBR<sup>TM</sup> Green Master Mix (Applied Biosystems) were used for RT-qPCR. The primers (Table 1) were synthesized by Eurofins Genomics (Huntsville, USA). The primer efficiency was calculated as described before (23) with the formula:  $E = 10^{(-1/\text{slope})}$ . The PCR was performed in a total volume of 20 µl; 10 µl of PowerUp<sup>TM</sup> SYBR<sup>TM</sup> Green Master Mix, 1.6 µl of forward primer (5 µM), 1.6 µl of reverse primer (5 µM), 5 µl of 1/10 diluted cDNA and 1.8 µl of molecular-grade water with the final primer concentration of 400 nM for all the genes except 16S (ribosomal RNA gene) and *rpoD* (sigma factor). For these two genes, a final primer concentration of 200 nM was used with reaction mixture, 10 µl of SYBR<sup>®</sup> Select Master Mix, 0.8 µl of forward primer (5 µM), 0.8 µl of reverse primer (5 µM), 5 µl of 1/10 diluted cDNA and 3.4 µl of molecular-grade water. Each PCR was performed in triplicate in the 96 well plates (MicroAmp<sup>TM</sup> Optical 96-Well Reaction Plate with Barcode, Fisher Scientific, Canada). PCR conditions were as follows: UDG activation at 50°C for 2 min and Dual-Lock<sup>TM</sup> DNA polymerase activation at 95°C for 2 min, followed by 40 repeated cycles of denaturation, annealing and amplification, at 95°C for 15 s, 54°C for 30 s and 72°C for 45 s. Subsequently, a default dissociation curve (95°C for 15 s, 60°C for 1 min and 95°C for 15 s) was performed in the instrument and specific amplicon was verified by a single melting-temperature peak. The transcript levels were normalized to the geometric average of expression for all housekeeping genes for each sample (24). The relative changes in gene expression were calculated by using the formula:  $dCT = CT(\text{target}) - CT(\text{Normalizer})$ , then  $ddCT$  is calculated by subtracting the  $dCT$  of the untreated sample from the treated one:  $ddCT = dCT(\text{treated}) - dCT(\text{untreated})$ . The relative gene expression is calculated as,  $2^{-ddCT}$ . Finally, a fold change was calculated as  $-1/2^{-ddCT}$  (25).

## Genome Sequencing of *L. helveticus* LH-2 and *L. acidophilus* LA-5

Genomic DNA was extracted from *L. helveticus* LH-2 and *L. acidophilus* La-5 by using the UltraClean Microbial DNA Isolation Kit (Mo-Bio Laboratories, Inc., Canada) according to

TABLE 1 | Oligonucleotides used in this study.

Gene	Function	Primer sequences	References
<i>hilA</i>	Transcriptional regulator of SPI-1	F: 5'-TGTCGGGAAGATAAAGAGCAT-3'R: 5'-AAGGAAGTATCGCCAATGTA-3'	(19)
<i>ssrB2</i>	Transcriptional regulator of SPI-2	F: 5'-TGGTTTACACAGCATACCAA-3'R: 5'-GGTCAATGTAACGCTTGTTF-3'	(19)
<i>16S</i>	16S rRNA gene, used as housekeeping gene	F: 5'-CAAGTCATCATGGCCCTTAC-3'R: 5'-CGGACTACGACGCACTTTAT-3'	(20)
<i>ropD</i>	Sigma factor, used as housekeeping gene	F: 5'-GTGAAATGGGCACTGTTGAACTG-3'R: 5'-TTCCAGCAGATAGGTAATGGCTTC-3'	(21)
<i>gmk</i>	Guanylate kinase, used as housekeeping gene	F: 5'-TTGGCAGGGAGCGCTTT-3'R: 5'-GCGCGAAGTGCCGTAGTA AT-3'	(22)

the manufacturer's instructions. DNA was eluted in 10 mM Tris-HCl (pH 8.0). The concentration and the purity of the purified DNA was measured at 260 and 280 nm using a Nanodrop 1000 spectrophotometer. The integrity of the DNA was verified by agarose (1%) gel electrophoresis. Extracted DNA samples were stored at  $-20^{\circ}\text{C}$ . Library preparation and sequencing were performed at the Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval Quebec, Canada). In short, the libraries were prepared using 500 ng of mechanically fragmented DNA by a Covaris M220 (Covaris) using the NEBNext Ultra II kit (New England Biolabs). TruSeq HT adapters (Illumina) were ligated instead of NEBNext adapters. The libraries were checked for quality using Bioanalyzer and quantified with Picogreen. The libraries were sequenced on a fraction of a MiSeq run (v3 600 cycles, Illumina) following the manufacturer's instructions. *De novo* assembly of the reads was performed using CLC Bio Genomic Workbench version 10.1 (Qiagen Inc., Mississauga, ON, Canada) at the Genomics Facility of the Advanced Analysis Centre, University of Guelph. Both the assembled reads and the *de novo* assembled contigs were BLAST searched (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Comparative genomic analysis of the proteolytic system was conducted using other related strains *L. helveticus* CNRZ 32 (abbreviation LAC LHE, accession number CP002081) for *L. helveticus* LH-2 and *L. acidophilus* NCFM (abbreviation LAC, accession number CP000033) for *L. acidophilus* La-5 using the NCBI microbial genome database and Blast alignment tools.

## Measurement of the Cell Envelope Proteinase (CEP) Activities

*L. helveticus* LH-2 and *L. acidophilus* La-5 were grown anaerobically in WPI—sugar based medium supplemented with 10 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  at  $37^{\circ}\text{C}$  for 24 h. The cells were harvested by centrifugation at  $15,000 \times g$  for 10 min at  $4^{\circ}\text{C}$  and washed twice with 10 mM  $\text{CaCl}_2$ -saline solution. The supernatant designated as extracellular extract (EE) was used for the measurement of cell lysis rate while the cells were re-suspended in Tris buffer (50 mM, pH 7.8) to an optical density at 600 nm ( $\text{OD}_{600}$ ) of approximately 10 and were used for CEP and aminopeptidase enzyme assays.

The CEP activity assay of intact cells was performed using the chromogenic substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma, Markham, ON, Canada) as described previously (26). The reaction mixture was consisted of 200  $\mu\text{l}$  resuspended cells, 287.5  $\mu\text{l}$  phosphate buffer (0.2 M, pH 7.0), 225  $\mu\text{l}$  5 M NaCl and 37.5  $\mu\text{l}$  N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (20 mM). The reaction components were mixed gently and incubated at different temperatures (35, 40, 45, 50, and  $55^{\circ}\text{C}$ ) for 30 min to determine the effect of temperature on the proteinase activity. The reactions were stopped by adding 175  $\mu\text{l}$  of 80% (v/v) acetic acid followed by centrifugation at  $13,000 \times g$  for 5 min. The release of p-nitroanilide (pNA) was measured at 410 nm using Beckman DU 500-Spectrophotometer. The enzyme activity was calculated using a molar absorption coefficient ( $\epsilon$ ) of  $8,800 \text{ mol}^{-1} \text{ cm}^{-1}$ . One unit of protease activity was defined as 1 nmol of p-nitroanilide released per minute. The

specific protease activity was calculated as one unit of protease from 1 mg of cell protein. The protein content was estimated using the method of Bradford (27). Bovine serum albumin (Thermoscientific, Rockford, USA) was used as a standard to measure the protein content.

## Aminopeptidase Activities

Suspensions of harvested cells with  $\text{OD}_{600}$  of approximately 10 were used for preparation of intracellular extracts (IE) according to Pescuma et al. (28), cells were disrupted by using Microbead tubes (Mo-Bio Laboratories, Inc., Canada) in a microbead tube vortex for 6 min at maximum speed with 1 min interruption on ice after each minute. Beads, cell debris and unbroken cells were removed by centrifugation ( $10,000 \times g$  at  $4^{\circ}\text{C}$  for 5 min) and the supernatant fluid was designated as the intracellular enzymatic extract (IE) which was maintained on ice and immediately used for enzymatic assays. Intracellular aminopeptidase activity was assayed with the chromogenic substrate Lys- $\rho\text{Na}$  (Sigma) as previously described (29) by incubation of 100  $\mu\text{l}$  of IE with 400  $\mu\text{l}$  of Tris-HCl buffer (50 mM, pH 7.0) and 50  $\mu\text{l}$  Lys- $\rho\text{Na}$  (10 mM) at five temperatures (35, 40, 45, 50, and  $55^{\circ}\text{C}$ ) for 20 min to determine the effect of temperature on the aminopeptidase activity. The reaction was stopped by addition of 1 mL of 30% acetic acid. The concentration of  $\rho$ -nitroanilide released was quantified at 410 nm. Enzyme activities were calculated by using a molar absorbance coefficient of  $9,024 \text{ mol}^{-1} \text{ cm}^{-1}$  under the assay conditions. One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of  $\rho$ -nitroanilide per min under the assay conditions. The specific peptidase activity was calculated as one unit of peptidase from 1 mg of cell protein. Aminopeptidase activity was measured in EE by the same procedures to detect rate of cell lysis.

## Identification of Peptides Through Liquid Chromatography–Mass Spectrometry Analysis (LC–MS)

Filtered CFSM was fractionated at three successive cut-off protein concentrator sizes 100, 50, and 3 kDa, respectively, using GE Healthcare Vivaspin™ 20 protein concentrator (Fisher Scientific). The filtrate was freeze dried for 72 h. A control sample of filter sterilized WPI CFSM was included. The freeze-dried filtrate of 30 ml CFSM was reconstituted in 300  $\mu\text{l}$  Milli-Q water and peptides were identified on an Agilent 1200 HPLC liquid chromatograph interfaced with an Agilent UHD 6530 Q-ToF mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph (30). Briefly, a C18 column (Agilent Advance Bio Peptide Map,  $100 \times 2.1 \text{ mm } 2.7 \mu\text{m}$ ) was used for chromatographic separation with the following solvents, water with 0.1% formic acid for A and acetonitrile with 0.1% formic acid for B. The mobile phase gradient was 2% B increasing to 45% B in 40 min and then to 55% B in 10 more minutes followed by column wash at 95% B and 10-min re-equilibration with a flow rate 0.2 mL/min.

The mass spectrometer electrospray capillary voltage was maintained at 4.0 kV and the drying gas temperature at  $350^{\circ}\text{C}$  with a flow rate of 13 L/min. Nebulizer pressure was 40 psi

and the fragmentor was set to 150. Nitrogen was used as both nebulizing and drying gas, and collision-induced gas. The mass-to-charge ratio was scanned across the  $m/z$  range of 300–2,000  $m/z$  in 4 GHz extended dynamic range positive-ion auto MS/MS mode. Three precursor ions per cycle were selected for fragmentation. The sample injection volume was 20  $\mu$ l. Raw data files were loaded directly into PEAKS 8 software (Bioinformatics Solutions Inc.) and subjected to *de novo* sequencing with SwissProt database searching. The tolerance values used were 15 ppm for parent ions and 0.05 Da for fragment ions. See **Supplementary Table S1** for information used to identify peptides.

## Statistical Analysis

All experiments were carried out three independent times with triplicates of each sample. Means and standard deviations were analyzed using ANOVA followed by Tukey's *post hoc* test with a  $P$ -value of  $\leq 0.05$  considered significant.

## RESULTS

### Activity of *L. helveticus* LH-2 and *L. acidophilus* La-5 CFSM on Bioluminescent Reporter Strains

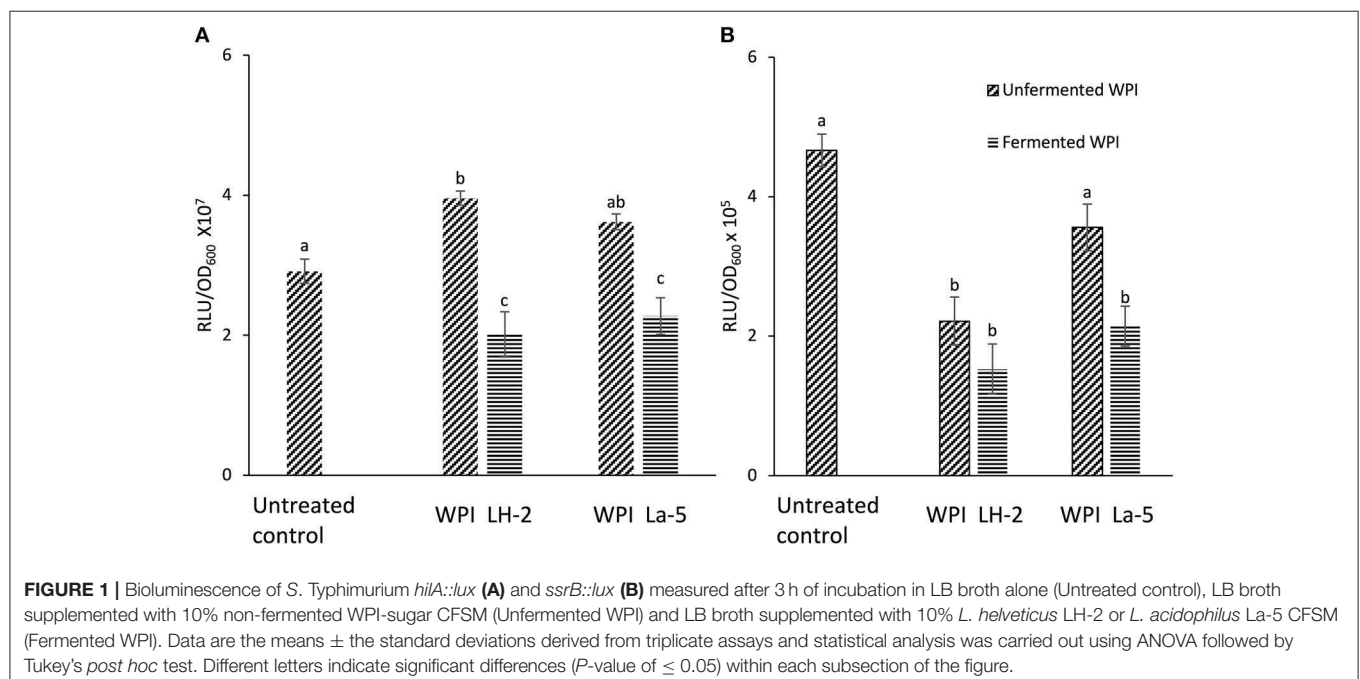
Bioluminescent reporter strains *S. Typhimurium hilA::lux* and *ssrB::lux* were used for initial screening of the antivirulence effects of CFSM obtained from WPI fermented with *L. helveticus* LH-2 and *L. acidophilus* La-5. CFSM from both strains reduced luminescence of the *S. Typhimurium hilA::lux* while only La-5 could decrease *ssrB::lux* reporter luminescence; indicating down-regulation of the *hilA* and *ssrB* genes (**Figures 1A,B**). Both uninoculated control WPI had no downregulatory effect

on *S. Typhimurium hilA::lux* light emission with a slight but significant increase in expression of the same gene in WPI glucose based media (**Figure 1A**). The uninoculated WPI sucrose based medium had no effect on *S. Typhimurium ssrB::lux* luminescence while WPI glucose based media showed significant down regulation of *S. Typhimurium ssrB::lux* luminescence (**Figure 1B**).

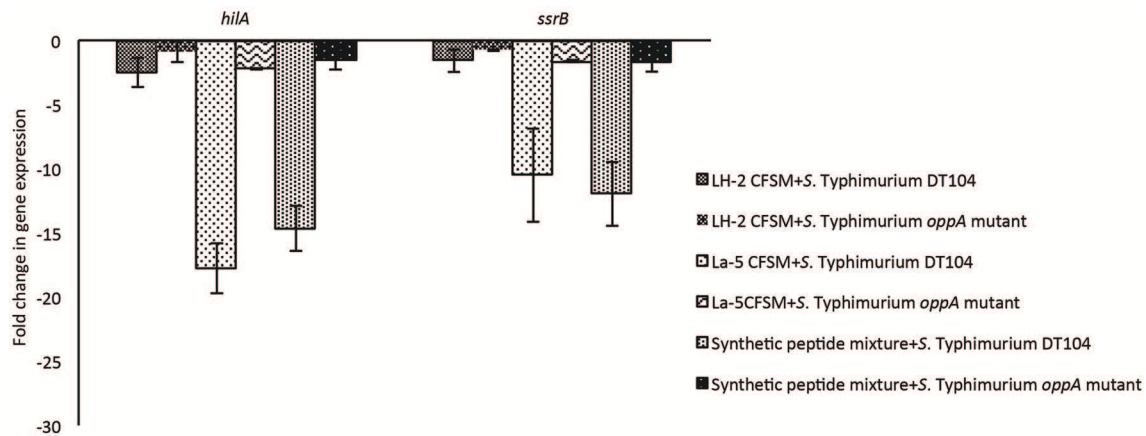
Bioluminescent reporter genes are considered a rapid tool for initial monitoring of gene expression. However, bioluminescence may be influenced by several factors, including other components of the medium. Previous observations using luminescent constructs suggest the occurrence of false positives and negatives with respect to virulence gene expression (12, 19, 31, 32). This is why an alternative method was chosen to complement the bioluminescence assay results. RT-qPCR is a highly sensitive and specific method for gene expression analysis.

### Effect of *L. helveticus* LH-2, *L. acidophilus* La-5 Bioactive Molecules and Mixture of Synthetic Peptides on Virulence Genes in *S. Typhimurium* DT 104 Wild Type and *S. Typhimurium oppA* Mutant Strains

The effects of the neutralized and reconstituted CFSM from *L. helveticus* LH-2 and *L. acidophilus* La-5 on virulence gene expression in multi-drug resistant *S. Typhimurium* DT104 and *S. Typhimurium oppA* mutant were analyzed by a 2-step RT qPCR method after incubation for 3 h (**Figure 2**). A statistically significant down-regulation of both the *hilA* and *ssrB* genes of *S. Typhimurium* DT 104 was observed after incubation with 10% CFSM from both LH-2 and La-5. La-5 CFSM showed stronger repression of *S. Typhimurium* DT104 *hilA* and *ssrB*







**FIGURE 2 |** Effect of *L. helveticus* LH-2 and *L. acidophilus* La-5 CFSM on virulence gene expression of *S. Typhimurium* DT104 and *S. Typhimurium oppA* mutant. Expression ratios of *hilA* and *ssrB* genes of *S. Typhimurium* were normalized to the expression of the geometric average of regulator genes *16S rRNA*, *rpoD*, *gmk* to obtain the fold-change and compared with those of the non-fermented media.

genes ( $-17.8$  and  $-10.49$ , respectively) than LH-2 CFSM ( $-2.56$  and  $-1.58$ , respectively). The expression of *hilA* and *ssrB* genes in *S. Typhimurium oppA* mutant strain was  $-2.23$  and  $-1.61$ , respectively, in the presence of La-5 CFSM while in the presence of LH-2 CFSM, expression of both genes was  $-0.92$  and  $-0.77$ , respectively (Table 2).

The synthetic peptides mixture could also downregulate the expression of *hilA* and *ssrB* genes in *S. Typhimurium* DT104 ( $-14.66$  and  $-11.99$ , respectively). The relative expression of the same genes in the *S. Typhimurium oppA* mutant strain in the presence of the same peptide mixture was  $-1.60$  and  $-1.67$ , respectively (Figure 2).

### Genes Associated With Oligopeptide Metabolism in *L. helveticus* LH-2 and *L. acidophilus* LA-5

*L. helveticus* CNRZ 32 and *L. acidophilus* NCFM have previously sequenced complete genomes with the most thoroughly characterized proteolytic system (15, 33). Whole Genome Sequencing (WGS) and comparative analysis of the proteolytic components of *L. helveticus* LH-2 and *L. acidophilus* La-5 with *L. helveticus* CNRZ 32 and *L. acidophilus* NCFM, respectively, show some genetic differences in the distribution of proteolytic elements in LH-2 and La-5 (Tables 3, 4). The *L. helveticus* LH-2 genome codes for *prtH3*, *prtH4*, and *prtM2* CEP genes (99, 99, and 100% nucleotide identity, respectively) with the absence of *prtH*, *prtH2* and *prtM* genes (Table 3). The *L. acidophilus* La-5 genome codes for *prtP* and *prtM* CEP genes (99, 100% identity, respectively) (Table 4).

Comparative genome analysis of *L. helveticus* LH-2 for oligopeptide transport elements and peptidases shows the presence of these proteolytic components with high identity (97–100%) (Table 3). The *L. acidophilus* La-5 genome codes for oligopeptide transport elements and peptidases of *L. acidophilus* NCFM with high identity (99–100%), except for *oppB2* (33% identity) (Table 4).

### CEP Activities of *L. helveticus* LH-2 and *L. acidophilus* LA-5 in WPI

The CEP activities of intact cells from the medium fermented by *L. helveticus* LH-2 and *L. acidophilus* La-5 were measured based on the *in vitro* hydrolysis of the chromogenic substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. The optimal temperature for the proteinase activity of LH-2 strain was  $45^{\circ}\text{C}$  with enzyme activity 3.60 while the maximum CEP activity of La-5 was  $0.25 \pm 0.01$  at  $40^{\circ}\text{C}$  (Table 5).

### Intracellular Aminopeptidase Activities of *L. helveticus* LH-2 and *L. acidophilus* LA-5 in WPI

For LH-2 intracellular extract, the highest aminopeptidase activity was  $22.06 \pm 0.60$  at  $40^{\circ}\text{C}$  while La-5 optimal activity of aminopeptidase was  $5.19 \pm 0.55$  at  $35^{\circ}\text{C}$  (Table 5). Negligible peptidase activity was detected in the extracellular supernatant using Lys-pNa, indicating that peptidase release from the cells during our experiments was below the detection limit (data not shown).

### Peptide Profiling of CSFM of Unfermented WPI and WPI Fermented With *L. helveticus* LH-2 and *L. acidophilus* LA-5 Strains

Liquid chromatography–mass spectrometry (LC-MS) analysis of unfermented WPI and WPI fermented with LH-2 and La-5 showed that peptides are almost all fragments of the main milk proteins (109, 75, and 15 milk protein derived peptides, respectively) (Figure 3). Out of these, 56 peptides were unique to the 3-kDa fraction from LH-2 fermented medium while 9 were unique to La-5 fermented medium. CEP cleavage sites are not evenly distributed throughout the protein sequences. Regions of highest proteolytic activity are demonstrated by heat maps of peptides (Figure 4). Thus, the presence of hot spots of cleavage sites are represented by changes in color scale, where



**TABLE 2 |** Effect of *L. helveticus* LH-2 and *L. acidophilus* La-5 CF5M on virulence gene expression of *S. Typhimurium* DT104 and *S. Typhimurium oppA* mutant compared with the control CF5M.

Genes	Target strain	PCR efficiency	Cycle threshold (Ct) values ± standard deviation					Synthetic peptide mixture
			No treatment	WPI-glucose CF5M	LH-2 CF5M	WPI-sucrose CF5M	La-5 CF5M	
16S	<i>S. Typhimurium</i> DT104	90.25%	6.46 ± 0.45	5.65 ± 0.33	6.61 ± 0.26	5.23 ± 0.09	5.32 ± 0.08	6.27 ± 0.17
	<i>S. Typhimurium oppA</i> mutant		7.00 ± 0.42	6.19 ± 0.28	6.22 ± 0.34	6.21 ± 0.42	6.08 ± 0.24	6.18 ± 1.23
<i>rpoD</i>	<i>S. Typhimurium</i> DT104	93.73%	22.58 ± 0.22	21.46 ± 0.20	22.07 ± 0.09	19.37 ± 0.21	20.75 ± 0.18	23.73 ± 0.47
	<i>S. Typhimurium oppA</i> mutant		22.21 ± 0.18	21.22 ± 0.18	21.11 ± 0.27	21.23 ± 0.21	21.45 ± 0.24	22.86 ± 2.78
<i>gmk</i>	<i>S. Typhimurium</i> DT104	101.69%	29.19 ± 0.82	26.95 ± 0.46	28.61 ± 0.33	25.59 ± 0.58	27.96 ± 0.08	32.28 ± 0.76
	<i>S. Typhimurium oppA</i> mutant		31.12 ± 0.76	29.16 ± 0.54	29.27 ± 2.23	29.63 ± 0.44	29.48 ± 0.64	32.38 ± 2.76
<i>hlyA</i>	<i>S. Typhimurium</i> DT104	96.37%	23.69 ± 0.36	25.31 ± 0.32	27.29 ± 0.14	20.74 ± 0.31	25.41 ± 0.17	25.94 ± 0.44
	<i>S. Typhimurium oppA</i> mutant		27.07 ± 0.25	27.08 ± 0.27	27.68 ± 0.48	27.74 ± 0.51	29.41 ± 0.31	30.96 ± 1.64
<i>ssrB2</i>	<i>S. Typhimurium</i> DT104	97.63%	26.29 ± 0.34	26.21 ± 0.22	27.41 ± 0.33	22.35 ± 0.06	26.67 ± 0.42	27.78 ± 0.46
	<i>S. Typhimurium oppA</i> mutant		26.14 ± 0.62	27.02 ± 0.26	27.05 ± 0.53	26.64 ± 0.79	27.03 ± 0.44	28.55 ± 2.41

**TABLE 3 |** Comparative genome analysis of the proteolytic system of *L. helveticus* LH-2 with *L. helveticus* CNRZ 32 (abbreviation LAC LHE, accession number CP002081) at the nucleotide level using NCBI microbial genome database and Blast alignment tools.

Gene (NCBI accession number)	Identity (%)
<b>CELL ENVELOPE-ASSOCIATED PROTEINASES</b>	
<i>prTH</i> (AAD50643)	0
<i>prTH2</i> (DQ826130)	0
<i>prTH3</i> (HQ602769)	4,913/4,914 (99%)
<i>prTH4</i> (HQ602770)	4,974/4,977 (99%)
<i>prtM</i> (DQ826131)	0
<i>prtM2</i> (DQ826132)	903/903 (100%)
<b>OLIGOPEPTIDE TRANSPORT</b>	
oligopeptide ABC transport protein ATP-binding component <i>OppF2</i> (AGQ24255.1)	314/314 (100%)
oligopeptide ABC transport protein ATP-binding component <i>OppD2</i> (AGQ24256.1)	351/352 (99%)
oligopeptide ABC transport protein ATP-binding component <i>OppF1</i> (AGQ23787.1)	328/328 (100%)
oligopeptide ABC transport protein ATP-binding component <i>OppD1</i> (AGQ23788.1)	342/344 (99%)
oligopeptide ABC transport protein periplasmic component <i>OppA4</i> (AGQ24260.1)	496/510 (97%)
oligopeptide ABC transport protein periplasmic component <i>OppA5</i> (AGQ24261.1)	538/540 (99%)
oligopeptide ABC transport protein ATP-binding component <i>OppB2</i> (AGQ24258.1)	309/309 (100%)
oligopeptide ABC transport protein permease component <i>OppC2</i> (AGQ24257.1)	269/269 (100%)
oligopeptide ABC transport protein permease component <i>OppC1</i> (AGQ23785.1)	285/286 (99%)
<b>PEPTIDASES</b>	
Oligoendopeptidase <i>pepF</i> (AY365129)	2,363/2,370 (99%)
Oligoendopeptidase <i>pepO</i> (AF019410)	2,690/2,691 (99%)
Oligoendopeptidase <i>pepO2</i> (DQ826126)	1,939/1,947 (99%)
sialoglycoprotein endopeptidase <i>Ogcp</i> (DQ826107)	1,050/1,050 (100%)
General aminopeptidases <i>pepCE</i> (JF811429)	1,314/1,314 (100%)
General aminopeptidases <i>pepC</i> (HQ602766)	1,349/1,350 (99%)
General aminopeptidases <i>pepN</i> (U08224)	3,754/3,787 (99%)
X-prolyl diaminopeptidase <i>pepX</i> (U22900)	3,204/3,204 (100%)
Proline iminopeptidases <i>pepI</i> (DQ826125)	885/885 (100%)
Proline iminopeptidases <i>pepR</i> (U05214)	1,259/1,260 (99%)
Prolidase <i>pepQ</i> (AF012084)	2,177/2,181 (99%)
Prolidase <i>pepQ2</i> (DQ826127)	1,110/1,110 (100%)
Dipeptidase <i>pepD</i> (U34257)	2,244/2,253 (99%)
Dipeptidase <i>pepD2</i> (DQ826122)	1,422/1,422 (100%)
Dipeptidase <i>pepD3</i> (DQ826123)	1,424/1,428 (99%)
Dipeptidase <i>pepV</i> (AF012085)	2,402/2,404 (99%)
Tripeptidase <i>pepT</i> (DQ826128)	1,242/1,242 (100%)
Tripeptidase <i>pepT2</i> (DQ826129)	1,287/1,287 (100%)
Glutamyl aminopeptidase <i>pepA</i> (DQ826138)	1,083/1,083 (100%)
Methionine aminopeptidase <i>map</i> (DQ826118)	828/828 (100%)
Pyrrolidone carboxyl peptidase <i>pcp</i> (DQ826121)	603/603 (100%)
SprT-like metallopeptidase <i>sprT</i> (HQ602765)	470/471 (99%)
M16 family metallopeptidase <i>hyp prt2</i> (DQ826112)	1,257/1,257 (100%)
Membrane alanine aminopeptidase <i>pepM1</i> (HQ602768)	1,515/1,515 (100%)

**TABLE 4 |** Comparative genome analysis of the proteolytic system of *L. acidophilus* La-5 with *L. acidophilus* NCFM (abbreviation LAC, accession code CP000033) at the nucleotide level using NCBI microbial genome database and Blast alignment tools.

Gene (NCBI accession number)	Identity (%)
<b>CELL ENVELOPE-ASSOCIATED PROTEINASES</b>	
<i>prtP</i> (LBA1512)	1,626/1,627 (99%)
<i>prtM</i> (LBA1588)	300/300 (100%)
<b>Oligopeptides transport:</b>	
Oligopeptide binding protein <i>oppA</i> (LBA0197)	1,626/1,626 (100%)
Oligopeptide ABC transporter, binding protein <i>oppA1B</i> (LBA0198)	542/542 (100%)
Oligopeptide ABC transporter, permease protein <i>oppB1</i> (LBA0200)	309/309 (100%)
Oligopeptide ABC transporter, permease protein <i>oppC1</i> (LBA0201)	343/343 (100%)
Oligopeptide ABC transporter, ATP binding protein <i>oppD1</i> (LBA0202)	352/352 (100%)
Oligopeptide ABC transporter, ATP binding protein <i>oppF1</i> (LBA0203)	313/314 (99%)
Oligopeptide ABC transporter, substrate binding protein <i>oppA3</i> (LBA1216)	527/527 (100%)
Oligopeptide ABC transporter, substrate binding protein <i>oppA2</i> (LBA1300)	584/585 (99%)
Oligopeptide ABC transporter, substrate binding protein <i>oppA2B</i> (LBA1301)	589/589 (100%)
Oligopeptide ABC transporter, permease protein <i>oppC2</i> (LBA1302)	309/309 (100%)
Oligopeptide ABC transporter, permease protein <i>oppB2</i> (LBA1303)	105/319 (33%)
Oligopeptide ABC transporter, ATP binding protein <i>oppF2</i> (LBA1305)	328/328 (100%)
Oligopeptide ABC transporter, ATP binding protein <i>oppD2</i> (LBA1306)	342/343 (99%)
Oligopeptide ABC transporter, substrate binding protein <i>oppA6</i> (LBA1400)	581/581 (100%)
Oligopeptide ABC transporter, substrate binding protein <i>oppA9</i> (LBA1961)	542/542 (100%)
<b>PEPTIDASES</b>	
Dipeptidase (LBA0035)	466/466 (100%)
Prolyl aminopeptidase (LBA0092)	293/293 (100%)
Metallopeptidase <i>pepO</i> (LBA0165)	649/650 (99%)
Pyrrolidone carboxyl peptidase (LBA0186)	200/200 (100%)
Dipeptidase <i>pepG</i> (LBA0195)	437/437 (100%)
Dipeptidase <i>pepE</i> (LBA0204)	438/438 (100%)
Prepilin peptidase (LBA0286)	228/229 (99%)
Aminopeptidase C <i>pepC</i> (LBA0343)	449/449 (100%)
Endopeptidase (LBA0390)	348/349 (99%)
Xaa-pro dipeptidase (LBA0430)	1,117/1,117 (100%)
<i>ampM</i> Methionine aminopeptidase (LBA0623)	274/275 (99%)
<i>pepC</i> Aminopeptidase (LBA0911)	437/437 (100%)
<i>pepD</i> Metallopeptidase (LBA0994)	467/467 (100%)
Signal peptidase (LBA1182)	256/257 (99%)
<i>pepT</i> Tripeptide aminopeptidase (LBA1190)	427/427 (100%)
<i>pepO</i> Metallopeptidase (LBA1275)	649/650 (99%)
<i>pepP</i> Xaa-Pro aminopeptidase (LBA1336)	1,124/1,124 (100%)

(Continued)

**TABLE 4 |** Continued

Gene (NCBI accession number)	Identity (%)
<i>pepQ</i> Metallopeptidase (LBA1343)	596/596 (100%)
<i>pepX</i> X-prolyl dipeptidyl aminopeptidase (LBA1373)	793/793 (100%)
<i>pepT</i> Tripeptide aminopeptidase (LBA1515)	427/427 (100%)
<i>vanY</i> d-alanyl-d-alanine carboxypeptidase (LBA1603)	1,312/1,312 (100%)
Prolyl aminopeptidase (LBA1658)	928/928 (100%)
<i>pepF</i> Oligoendopeptidase (LBA1763)	598/598 (100%)
<i>pepN</i> Membrane alanyl aminopeptidase (LBA1849)	844/844 (100%)
Signal peptidase (LBA1909)	210/210 (100%)
<i>pepL</i> Leucyl aminopeptidase (LBA1957)	299/299 (100%)

red represents more numerous peptides. The undigested WPI shows many plasmin and cathepsin cleavage sites (Figures 5–8). Endogenous milk peptides were mostly not found after fermentation with LH-2 and La-5 strains, except 14 peptides from  $\beta$ -casein and one peptide from  $\alpha_{s2}$ -casein (Figure 3). Most peptides found in the supernatant of LH-2 and La-5 fermented media display low-affinity residues for oligopeptide binding proteins (negatively charged amino acids, peptides with glycine, proline and glutamine at position 4, 5, or 6, peptides with proline at second position, VPP and IPP containing peptides and phosphorylated peptides) and higher resistance to peptidase hydrolysis (Figures 5–9).

Identification of bioactive peptide sequences was conducted through a search of the peptide literature databases (Table 6). Out of the 109 peptides in undigested WPI, 39 peptides have previously reported bioactivity (antihypertensive, antimicrobial, antioxidative, immunomodulatory, antithrombotic, opioid and antidiabetic activity). A total of 75 peptides were found in *L. helveticus* LH-2 CFMS, 19 of these peptides have previously been associated with several categories of bioactivity (ACE-inhibitory, opioid, antimicrobial and antioxidant activities). For *L. acidophilus* La-5 CFMS, a total of 15 peptides were sequenced, 5 of these peptides have previously been reported with ACE-inhibitory, antioxidant, antimicrobial and anti-caries activities. Some of these peptides have multifunctional properties that can modulate two or more physiological processes (Table 6).

## DISCUSSION

Several previous studies showed the ability of lactic acid bacteria to down regulate the expression of virulence genes of enteropathogenic bacteria (31, 86, 87). In this study, CFMS collected from WPI fermented by *L. helveticus* LH-2 and *L. acidophilus* La-5 reduced the expression of both the *hilA* and *ssrB* genes of the *S. Typhimurium* DT 104 wild strain. *L. acidophilus* La-5 has the most significant down-regulatory effect on the virulence genes, which indicates that the antivirulence effect is strain dependent and affected by the nature and components of CFMS. WPI fermented by La-5 contains 9 unique peptides not found in LH-2-fermented or unfermented medium.

Delcenserie et al. (32) reported that glucose could up or down-regulate the expression of virulence genes in *E. coli*

**TABLE 5** | Specific proteinase and aminopeptidase activities of intact cells and intracellular enzymatic extract of *L. helveticus* LH-2 and *L. acidophilus* La-5 after growth anaerobically in WPI—sugar based media at 37°C for 24 h.

Temperature (°C)	Specific proteinase activity		Specific aminopeptidase activity	
	<i>L. helveticus</i> LH-2	<i>L. acidophilus</i> La-5	<i>L. helveticus</i> LH-2	<i>L. acidophilus</i> La-5
35	1.66 ± 0.04	0.05 ± nd	19.74 ± 0.27	5.19 ± 0.55
40	2.12 ± 0.11	0.25 ± 0.01	22.06 ± 0.60	4.08 ± 0.74
45	3.60 ± nd	0.14 ± 0.01	7.82 ± 0.91	4.43 ± 0.37
50	3.44 ± 0.03	0.21 ± 0.01	3.41 ± 0.23	2.15 ± 0.72
55	3.01 ± 0.03	0.06 ± 0.01	14.78 ± 1.32	2.75 ± 0.36

Values are the means and SD of independent triplicates. non- detectable (nd) = SD < 0.01. Specific enzyme activity is expressed as nmoles pNa released /mg protein/min.

O157:H7. In our study, glucose and sucrose may also affect virulence gene expression in *S. Typhimurium*. A peptidic fraction, isolated from milk fermented with *L. helveticus*, down-regulated *ssrB* gene expression of *S. Typhimurium* in the absence of glucose in the growth medium (10). CFMSM from MRS without glucose fermented with some bifidobacteria species showed down-regulation of genes *hilA*, *ssrB2*, and *sopD* (88). These studies suggest that down-regulation of virulence genes could be caused by other non-carbohydrate metabolites produced after fermentation.

Absence of the downregulatory effect of *L. helveticus* LH-2 and *L. acidophilus* La-5 CFMSM on the *Salmonella oppA* mutant strain signifies the importance of peptide transport inside *S. Typhimurium* to inhibit virulence gene expression. Regulation of virulence gene expression requires sensing of a specific signal in the environment such as autoinducers for quorum sensing (QS) and these signals affect the growth, metabolism and virulence of bacteria (89). Exogenous leucine increases the transport of peptides by the Opp system and enhances OppA synthesis (90). Also, activity of Opp increases the intracellular amino acid pool, which in turn activates global regulators such as Lrp or CodY (91). Baek et al. (92) reported that Lrp responds to the nutritional environment and has a repressor function on key virulence regulator genes of *S. enterica* Serovar Typhimurium by strong interaction with *hilA* and other SPI-1 and SPI-2 genes, affecting their expression by binding directly to their promoter regions  $P_{hilA}$ ,  $P_{invF}$  and  $P_{ssrA}$ . From these studies and our data, it can be concluded that the presence of the *S. Typhimurium oppA* gene is a key factor in sensing oligopeptides, which can act as a specific signal for down regulation of virulence genes once transported into the cell.

The downregulatory effect of the synthetic peptide mixture demonstrates the antivirulence effect of specific peptides produced by *L. helveticus* LH-2 and *L. acidophilus* La-5 CFMSM on the *S. Typhimurium* virulence genes. Also, the absence of this downregulatory effect for the *S. Typhimurium oppA* mutant strain shows the importance of internalization of such peptides inside *S. Typhimurium* to exhibit the antivirulence effect.

Presence of more than one CEP paralog is common among *L. helveticus* strains (93). Also, the presence of *prtH3* and *prtH4* with the absence of *prtH* in *L. helveticus* LH-2 is not unusual as *prtH* is not broadly distributed within *L. helveticus* and has no

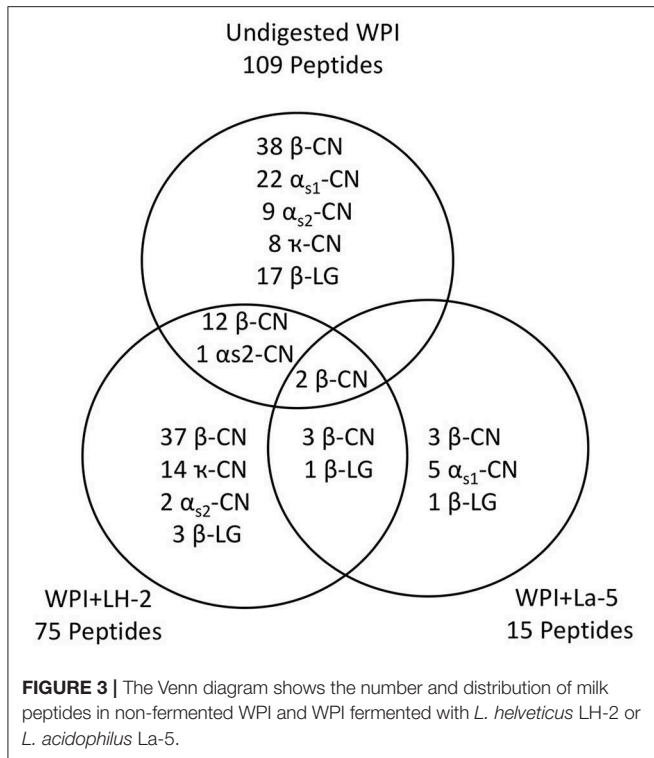
effect on growth rate in milk and cheese whey (94). *prtH* and *prtH2* distribution is also strain dependent (95). The *prtH3/prtH4* combination is common in *L. helveticus* strains (96). These studies agree with our findings for the presence of *prtH3* and *prtH4* genes and absence of *prtH* and *prtH2* genes in the genome of the LH-2 strain. *PrtM* is required for activation of *PrtH* and *PrtM2* plays a role in activation of other CEP paralogs in *L. helveticus* (97). Thus, absence of *PrtM* in LH-2 is expected as this gene was almost exclusively restricted to strains that also contained *prtH* (33).

Presence of both *PrtP* (proteinase precursor) and *PrtM* (maturase) in *L. acidophilus* La-5 confirmed that La-5 can digest large proteins extracellularly and produce small peptides. Genay et al. (95) reported that the genetic biodiversity of CEP paralogs between LAB strains could affect growth rate, strain functionality and bioactive peptide production in dairy products. This could cause differences in the antivirulence activities and bioactive peptides between of LH-2 and La-5 after growth in WPI.

Peptide transport systems can be specific for oligopeptides, or di- and tri-peptides (98). *L. helveticus* LH-2 contains all oligopeptide ABC transport elements, which is not different from previous studies of *L. helveticus* strains (99). *L. acidophilus* La-5 shows low identity of *oppB2* which is encoded by the *opp2*-type operon (100). Azcarate-Peril et al. (101) reported that Opp1 and Opp2 might have different specificities, which could explain accumulation of some peptides in La-5 growth media. Doeven et al. (98) also concluded that the lack of some elements of the membrane complex, OppBCDF, leads to presence of some peptides outside the cells due to the specificity of the transport process.

The functionality of these enzymes after genome analysis of *L. helveticus* LH-2 and *L. acidophilus* La-5 strains was determined by measuring the proteolytic enzyme activities. The optimal temperature for the proteinase activity of LH-2 (45°C) is not different from other previously reported *L. helveticus* strains (40–50°C) (102–104). The maximum CEP activity of La-5 (40 and 50°C) also is not significantly different from other *L. acidophilus* strains (45–50°C) previously reported (105).

*L. helveticus* LH2 CEP activity in WPI was lower than the CEP activity of other *L. helveticus* strains previously reported in skim milk (104, 106). The CEP activity of La-5 was slightly lower than the CEP activity of the same strain in skim milk (106) and significantly lower than the CEP activities of other *L. acidophilus*

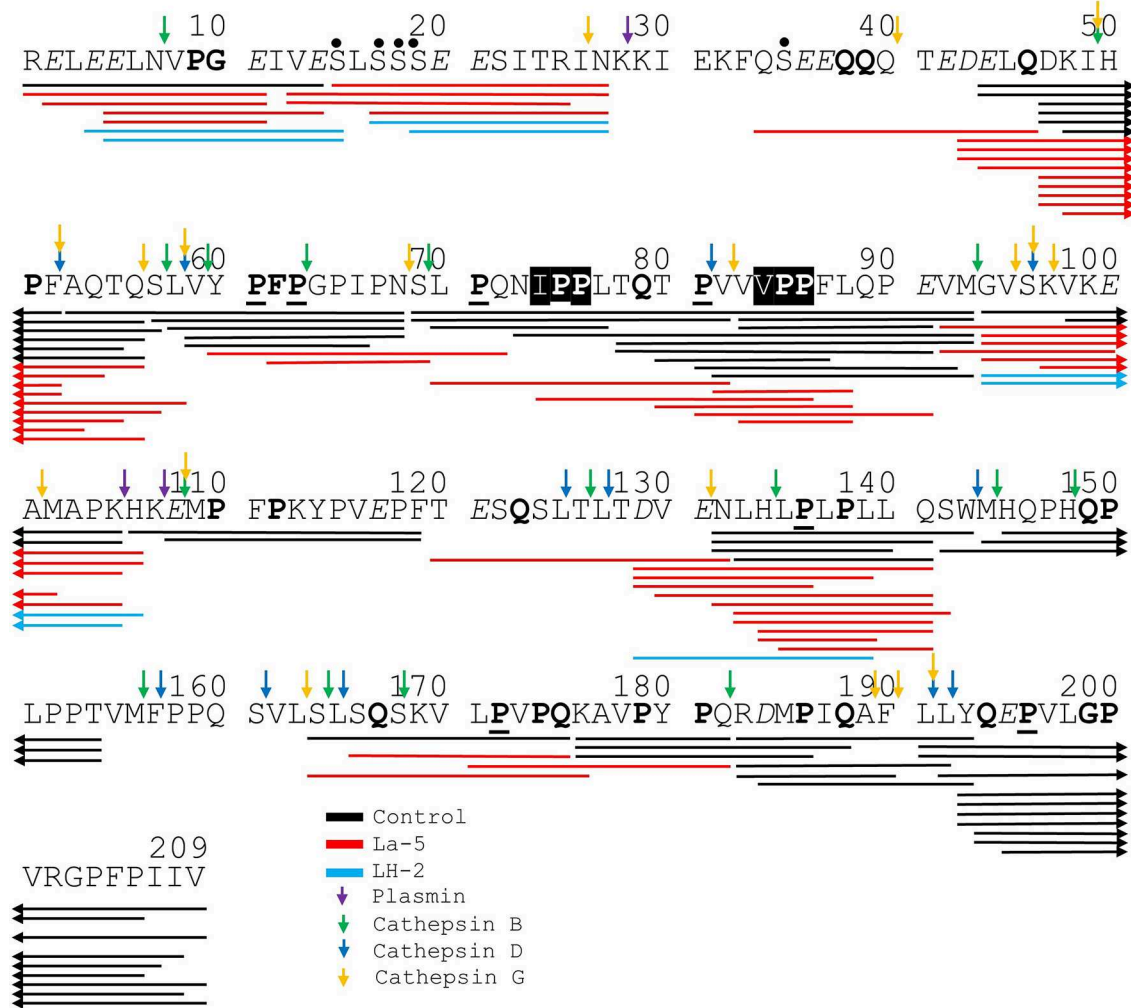


strains in chemically defined medium (107). The presence of peptides in the growth medium could inhibit the proteinase activity of some LAB (108). Mass spectrometry analysis of non-fermented WPI in this study confirmed the presence of endogenous milk protein derived peptides ( $\beta$ -,  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\kappa$ -casein, and  $\beta$ -lactoglobulin) after ultrafiltration of milk during the WPI production process. The presence of these endogenous peptides in the growth media could explain the lower CEP activities of LH-2 and La-5 in WPI compared with other related strains in milk-based growth media. The CEP activity levels of lactobacilli depend on the strain, nature and quantity of peptides present in the growth media (96). The regulation of proteinase activity is also strain dependent (107). These factors suggest that CEP activities of LH-2 and La-5 are different in WPI than other milk-based media.

*L. helveticus* LH-2 aminopeptidase activity in WPI based medium was significantly lower compared with previously studied *L. helveticus* strains after growth in simplified chemically defined medium (SCDM) supplemented with different nitrogen sources (109). *L. acidophilus* La-5 had higher aminopeptidase activity compared with *L. acidophilus* strains mentioned in Pescuma et al. (28) when grown in a chemically defined medium (CDM). The aminopeptidase activities are not influenced by the peptide content of the medium (109), so this difference may be mainly strain dependent (110).





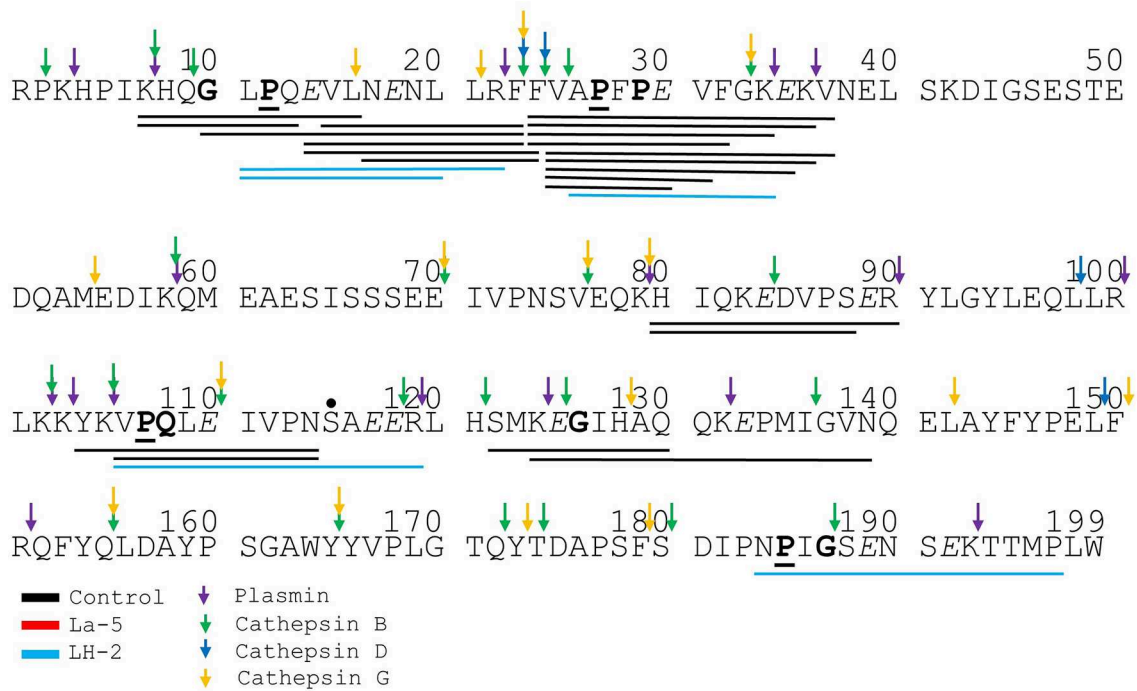


**FIGURE 5** | Bovine  $\beta$ -casein derived peptides identified in non-fermented WPI and WPI fermented with *L. helveticus* LH-2 and *L. acidophilus* La-5. Negative charged amino acids (Aspartate D, glutamate E) are in italics, Glycine G, Proline P, and Glutamine Q at position 4, 5, or 6 are marked with bold. P at second position is marked with bold and underline. VPP and IPP are inverse colors (white characters on black background). Phosphorylation sites are marked with a black dot above the serine. Cleavage sites of endogenous milk proteases are adapted from Baum et al. (34) and marked with colored vertical arrows.

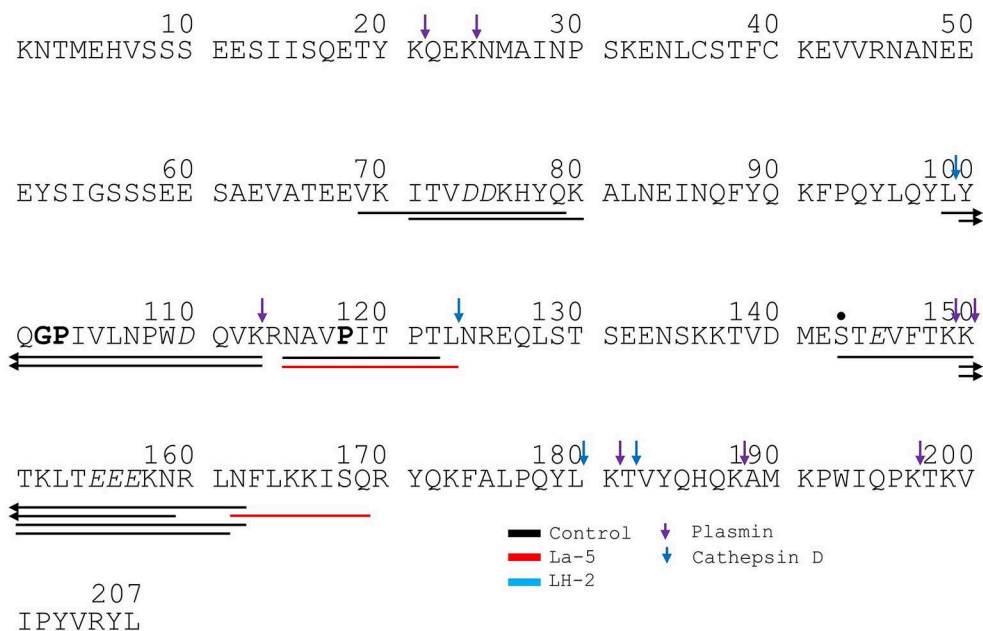
The presence of endogenous peptides in unfermented WPI indicates the activity of endogenous milk proteases which are mainly plasmin, elastase and cathepsin D, B, and G (111). Plasmin has little or no activity toward  $\kappa$ -casein and whey proteins (112), so the presence of endogenous peptides from these proteins in CFSM of *L. acidophilus* La-5 and *L. helveticus* LH-2 could be due to the action of other milk proteases such as cathepsin B, D, and G (34, 113). Dallas et al. (114) concluded that minimal proteolysis by native milk enzymes continued to function during incubation in the heat-treated milk when compared with that carried out by the proteases of kefir microorganisms which were mainly *L. acidophilus* and *L. helveticus*. This observation could explain the presence of some peptides shared between the unfermented and fermented WPI. Absence of some peptides after WPI fermentation is likely due to either further hydrolysis by LH-2 and La-5 extracellular proteases or their uptake by these

microorganisms. The presence of four shared peptides (three from  $\beta$ -casein and one from  $\beta$ -lactoglobulin) in WPI fermented by LH-2 and La-5 strains indicates some similarity in the affinity of the proteinases of these strains for certain cleavage sites.

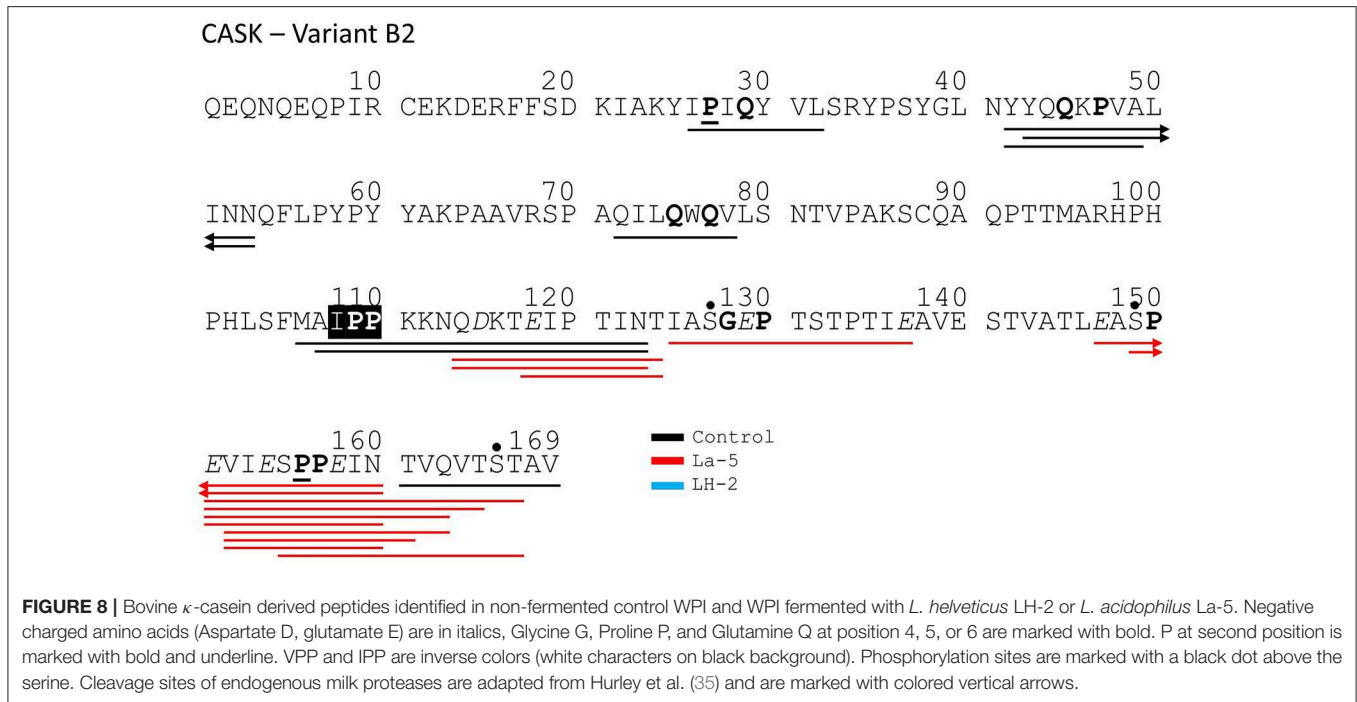
According to peptide analysis data, most of the identified peptides are casein derived, even though whey was used. The presence of casein derived peptides indicates that these proteins were exposed to proteolytic cleavage during processing (115). The three dimensional structure of caseins is more open and flexible than the globular, rigid, compact structures of whey proteins, which make casein proteins more susceptible to the effect of proteases (116). Whey proteins also are not an important peptide precursor during initial fermentation or endogenous proteolysis (117). This could explain why most peptides in the unfermented and fermented WPI media originate from casein proteins.



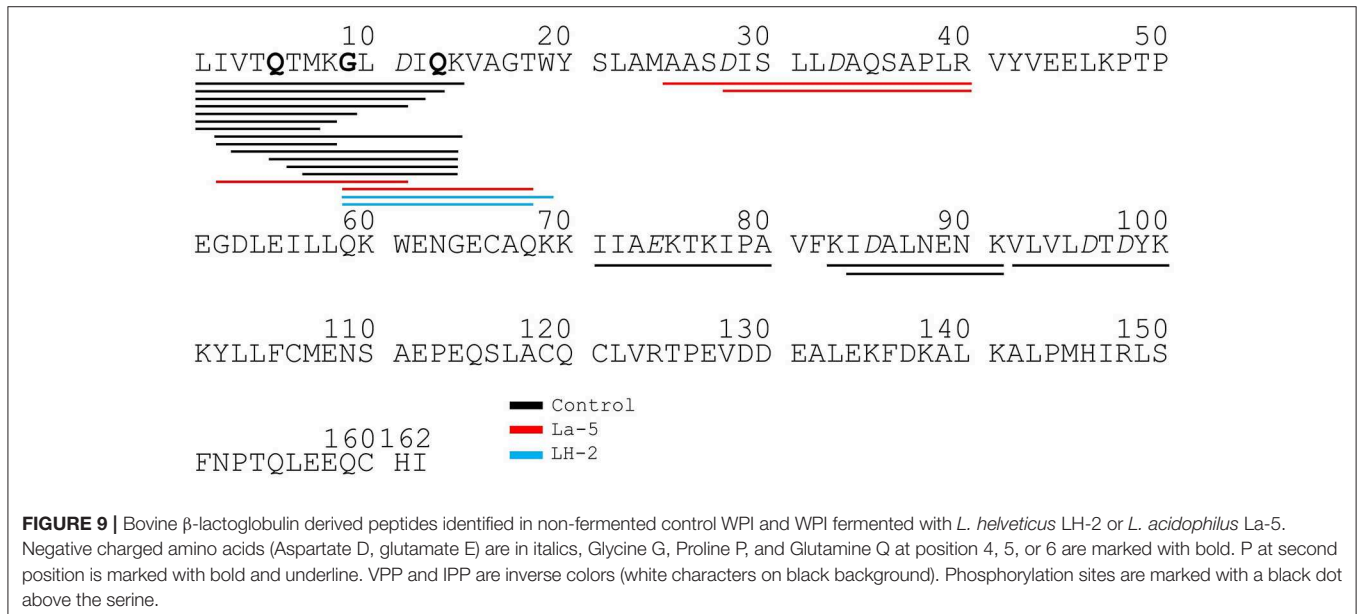
**FIGURE 6 |** Bovine  $\alpha_{s1}$ -casein derived peptides identified in non-fermented WPI and WPI fermented with *L. helveticus* LH-2 and *L. acidophilus* La-5. Negative charged amino acids (Aspartate D, glutamate E) are in italics, Glycine G, Proline P, and Glutamine Q at position 4, 5, or 6 are marked with bold. P at second position is marked with bold and underline. VPP and IPP are inverse colors (white characters on black background). Phosphorylation sites are marked with a black dot above the serine. Cleavage sites of endogenous milk proteases are adapted from Baum et al. (34) and are marked with colored vertical arrows.



**FIGURE 7 |** Bovine  $\alpha_{s2}$ -casein derived peptides identified in non-fermented WPI and WPI fermented with *L. helveticus* LH-2 or *L. acidophilus* La-5. Negative charged amino acids (Aspartate D, glutamate E) are in italics, Glycine G, Proline P, and Glutamine Q at position 4, 5, or 6 are marked with bold. P at second position is marked with bold and underline. VPP and IPP are inverse colors (white characters on black background). Phosphorylation sites are marked with a black dot above the serine. Cleavage sites of endogenous milk proteases are adapted from Baum et al. (34) and are marked with colored vertical arrows.



**FIGURE 8 |** Bovine  $\kappa$ -casein derived peptides identified in non-fermented control WPI and WPI fermented with *L. helveticus* LH-2 or *L. acidophilus* La-5. Negative charged amino acids (Aspartate D, glutamate E) are in italics, Glycine G, Proline P, and Glutamine Q at position 4, 5, or 6 are marked with bold. P at second position is marked with bold and underline. VPP and IPP are inverse colors (white characters on black background). Phosphorylation sites are marked with a black dot above the serine. Cleavage sites of endogenous milk proteases are adapted from Hurley et al. (35) and are marked with colored vertical arrows.



**FIGURE 9 |** Bovine  $\beta$ -lactoglobulin derived peptides identified in non-fermented control WPI and WPI fermented with *L. helveticus* LH-2 or *L. acidophilus* La-5. Negative charged amino acids (Aspartate D, glutamate E) are in italics, Glycine G, Proline P, and Glutamine Q at position 4, 5, or 6 are marked with bold. P at second position is marked with bold and underline. VPP and IPP are inverse colors (white characters on black background). Phosphorylation sites are marked with a black dot above the serine.

Although  $\beta$ -casein is not the most abundant protein in casein, our data showed that about half (47, 72, and 53%) of identified peptides in the WPI and WPI fermented with LH-2 and La-5, respectively, were derived from  $\beta$ -casein. Thus, it can be concluded that the endogenous and microbial proteases of LH-2 and La-5 may preferentially attack  $\beta$ -casein. These findings are consistent with previously studies on bovine casein and kefir (49). The degradation of  $\alpha$ s-casein is strain dependent (107). Our results confirm this finding, as La-5 shows different cleavage sites for  $\alpha$ s1-casein while  $\alpha$ s1-casein derived peptides were not found

after fermentation with LH-2. Nielsen et al. (67) reported no peptides from  $\alpha$ s1-casein digested with *L. helveticus* 1198 while Sadat-Mekmene et al. (118) concluded that  $\alpha$ s1-casein hydrolysis was enhanced in the presence of both CEPs (*prtH*, *prtH2*). The lack of the *prtH* CEP gene in the *L. helveticus* LH-2 genome does not explain the absence of  $\alpha$ s1-casein derived peptides in LH-2 CFMS, as some of these are present in the control medium. Strain LHC2, which has a similar combination of CEP homologs (PrtH3/PrtH4), does produce peptides from  $\alpha$ s1-casein within a 3-h time scale (119). The longer 48-h time scale of the

**TABLE 6 |** Bioactive peptides identified in Control WPI and WPI fermented with *L. helveticus* LH-2 and *L. acidophilus* La-5 strains by searching the Milk Bioactive Peptide Database with the reported function of the identified peptides.

Protein	Peptide	Function	Control	LH-2	La-5	References	
Bovine $\beta$ -CN	RELEELNVPGEIVE	ACE-inhibitory	X			(36)	
	ELQDKIHPF	ACE-inhibitory	X			(37)	
	SLVYFPFGPIPN	ACE-inhibitory	X			(38)	
	LVYFPFGPIPN	ACE-inhibitory	X			(39)	
	VYFPFGPI	Prolyl endopeptidase-inhibitory	X			(40)	
	VYFPFGPIPN	Antioxidant, ACE-inhibitory	X			(41)	
	SLPQNIPPLTQTPV	ACE-inhibitory	X			(42)	
	SLPQNIPPLTQTPVWPPFLQPEVM	ACE-inhibitory	X			(36)	
	LPQNIPPL	Antidiabetic	X			(43)	
	TQTPVWPPFLQPE	Antioxidant	X			(44)	
	GVSKVKEAMAPK	Antioxidant	X	X	X	(45)	
	VKEAMAPK	Antibacterial, Antioxidant, ACE-inhibitory	X			(45–47)	
	HKEMPFKYPVEPF	Opioid	X			(48)	
	EMPFKYPVEPF	ACE-inhibitory	X			(49)	
	NLHLPLPLLQ	ACE-inhibitory	X	X		(50)	
	MHQPHQPLPPT	Antitrotaviral Activity	X			(51)	
	HQPHQPLPPT	Immunomodulatory	X			(52)	
	SLSQSKVLPVPQ	Antioxidant	X			(53)	
	SQSKVLPVPQ	ACE-inhibitory	X	X		(54)	
	RDMPIQAF	ACE-inhibitory	X			(42)	
	LLYQEPVLGPRGPFPIIV	ACE-inhibitory, Immunomodulatory, Antithrombin, Antimicrobial	X			(55–57)	
	LYQEPVLGPRGPFPIIV	Mitogen	X			(58)	
	YQEPVLGPRGPFPI	ACE-inhibitory	X			(59)	
	YQEPVLGPRGPFPI	Antimicrobial	X			(56, 60)	
	YQEPVLGPRGPFPIIV	Immunomodulatory, Antimicrobial	X			(61)	
	QEPVLGPRGPFPIIV	ACE-inhibitory, Antioxidant	X			(59, 62)	
	EPVLGPRGPFPIIV	ACE-inhibitory	X			(45)	
	DKIHPFA	ACE-inhibitory			X	(63)	
	YFPFGPIHNSLPQ	Opioid			X	(64)	
	LPQNIPPLTQTPV	Antidiabetic, ACE-inhibitory			X	(43, 65)	
	IPPLTQTPVWPP	ACE-inhibitory			X	(66)	
	TPVWPPFL	ACE-inhibitory			X	(37, 67)	
	PVWPPFLQPE	Antimicrobial			X	(60)	
	DVENLHLPLPL	ACE-inhibitory			X	X	(68)
	LHLPLPLLQS	ACE-inhibitory			X		(69)
	HLPLPLLQ	Enteric nervous system development			X		(70)
SLSQSKVLPVPQK	Antioxidant			X		(53)	
LPVPQKAVYPQ	Antioxidant			X		(71)	
Bovine $\alpha_{S1}$ -CN	EVLNENLLRF	ACE-inhibitory	X			(72)	
	FVAPFPEVFGK	ACE-inhibitory	X			(73)	
	VAPFPEVFGKE	ACE-inhibitory	X			(74)	
Bovine $\alpha_{S2}$ -CN	VPQLEIVPNSAEER	Mineral carriers, anti-caries activity			X	(75)	
	LYQGPIVLNPWDQVK	ACE-inhibitory	X			(65)	
Bovine $\alpha_{S2}$ -CN	NAVPIPT	ACE-inhibitory	X			(65)	
	NAVPIPTL	Antioxidative		X		(76)	
Bovine $\kappa$ -CN	YYQQKPA	Antibacterial	X			(77)	
	MAIPPKKNQDKTEIPTIN	Antithrombotic, Antibacterial	X			(78)	
	TVQVTSTAV	Antibacterial	X			(77, 78)	

(Continued)



TABLE 6 | Continued

Protein	Peptide	Function	Control	LH-2	La-5	References
	EIPTINT	Antibacterial		X		(77)
	EVIESPPEINTVQVT	ACE-inhibitory, zinc-chelation		X		(79)
Bovine $\beta$ -LG	IIAEKTKIPA	ACE-inhibitory	X			(80)
	LIVTQTMK	Cytotoxic	X			(81)
	IDALNENK	Antibacterial	X			(82)
	VLVLDTDYK	Antibacterial, DPP-IV inhibitory	X			(60, 83)
	AASDISLLDAQSAPLR	Antibacterial		X		(84)
	GLDIQKVAGT	ACE-inhibitory, Antibacterial		X	X	(60, 85)
	GLDIQKVAGTW	ACE-inhibitory			X	(85)

fermentation of WPI by LH-2 may explain the disappearance of  $\alpha$ s1-casein peptides, and the lack of larger casein proteins in the WPI medium would preclude the release of additional  $\alpha$ s1-casein peptides by LH-2. In contrast, LH-2 can produce  $\alpha$ s2-casein derived peptides while La-5 cannot produce them. Pescuma et al. (107) reported that *L. acidophilus* strain CRL 636 was unable to degrade  $\alpha$ s2-casein, which supports the current findings. LH-2 shares some  $\kappa$ -casein cleavage sites with other *L. helveticus* strains (120) while  $\kappa$ -casein is poorly degraded by La-5 (105).  $\beta$ -lactoglobulin is one of the major milk allergens (121). LH-2 and La-5 strains were able to degrade this protein, which indicates the potential of these strains in the production of hypoallergenic dairy products.

Fira et al. (105) reported that the optimal pH for CEP of *L. acidophilus* strains tested was 6.5 with a temperature optimum of 45–50°C, which might explain the lower number or diversity of peptides produced by La-5 at a lower pH and temperature (pH 4.5–5.2 at 37°C). The La-5 genome codes for only one CEP when compared with LH-2, which could result in a lower number or diversity of released peptides accumulating in the growth medium. Even though La-5 produces fewer types of peptides, those that are produced have higher antivirulence activity compare to those produced by LH-2, which depends on the nature and structure of the peptides. None of the peptides found in WPI fermented by La-5 are similar to those found in previous studies of the same strain grown in skim milk (52), which emphasizes the significant effect of growth media on the proteolytic activity of LAB.

Accumulation of peptides with predicted antivirulence effect after fermentation despite the active proteolytic and peptidolytic enzymes of *L. helveticus* LH-2 and *L. acidophilus* La-5 may be due to the nature and structure of these peptides. Detmers et al. (122) reported that peptides with hydrophobic and aromatic residues have high binding affinity to OppA, while those with proline, glycine, negatively charged amino acids (Aspartate D, glutamate E) and peptides with neutral or positively charged N-terminal residues having Gly, Pro and/or Gln in position 4, 5 and/or 6 significantly lower the affinity of the peptide for OppA protein. Proline at the second position of a nonameric peptide also resulted in a dramatic drop of the OppA binding affinity (122). Phosphorylated peptides and peptides high in proline content are more resistant to hydrolysis by proteolytic enzymes (123, 124).

Oligopeptides containing VPP and IPP sequences could release branched chain amino acids (BCAAs) in the presence of specific peptidases. BCAAs could enhance binding of the Branched Chain Amino Acids Responsive Transcriptional Regulator (BCARR) to DNA sequences in the upstream region of the *pepV* gene, which would repress the expression of some peptidase genes in *L. helveticus* (125). Most LH-2 and La-5 peptides found in the supernatant display some of these characteristics, which may help explain their accumulation in the fermented WPI medium.

## CONCLUSIONS

*L. helveticus* LH-2 and *L. acidophilus* La-5 produce peptides with antivirulence effect against *Salmonella enterica* subsp. *enterica* serovar Typhimurium after growth in whey protein isolate medium. Accumulation of peptides with antivirulence activities may be related to the composition of these peptides and low affinity of these peptides to the oligopeptide-binding protein (OppA) of these strains, thus remaining in the spent medium if they are not transported into the cell. In *Salmonella*, the antivirulence activity of milk protein-derived peptides is related to the presence of the *oppA* gene. The undigested and fermented WPI by LH-2 and La-5 strains could be considered as a possible source of natural and functional ingredients, which may be used to increase the biological activity of food products. Further studies are required to explore the antivirulence ability of individual synthetic peptides with the same sequence at different concentrations compared to the antivirulence activity of fermented WPI.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the the University of Guelph Research Data Repository [https://doi.org/10.5683/SP2/43M6GX].

## AUTHOR CONTRIBUTIONS

EA carried out the experiments, interpreted the results, and wrote the first draft of the manuscript. SN participated in interpretation

and presentation of the peptide results. SA-E, AE-L, and ES provided help for interpretation of the results. GL was involved in the design of the study, interpretation of the results, and writing of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2019.00152/full#supplementary-material>

**Supplementary Table S1** | Information used to identify peptides in unfermented control medium and media fermented by strain La-5 or LH-2.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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