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Special Issue Article

Cell factory applications of the yeast *Kluyveromyces marxianus* for the biotechnological production of natural flavour and fragrance molecules

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Abstract

Kluyveromyces marxianus is emerging as a new platform organism for the production of flavour and fragrance (F&F) compounds. This food-grade yeast has advantageous traits, such as thermotolerance and rapid growth, that make it attractive for cell factory applications. The major impediment to its development has been limited fundamental knowledge of its genetics and physiology, but this is rapidly changing. *K. marxianus* produces a wide array of volatile molecules and contributes to the flavour of a range of different fermented beverages. Advantage is now being taken of this to develop strains for the production of metabolites such as 2-phenylethanol and ethyl acetate. Strains that were selected from initial screens were used to optimize processes for production of these F&F molecules. Most developments have focused on optimizing growth conditions and the fermentation process, including product removal, with future advancement likely to involve development of new strains through the application of evolutionary or rational engineering strategies. This is being facilitated by new genomic and molecular tools. Furthermore, synthetic biology offers a route to introduce new biosynthetic pathways into this yeast for F&F production. Consumer demand for biologically-synthesized molecules for use in foods and other products creates an opportunity to exploit the unique potential of *K. marxianus* for this cell factory application. Copyright © 2014 John Wiley & Sons, Ltd.

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Introduction to *Kluyveromyces marxianus*

Kluyveromyces marxianus is the type species of the genus *Kluyveromyces*, which, following several reclassifications, currently contains six described species (Kurtzman, 2003; Lachance, 2007). In the scientific literature, *K. marxianus* has also been described by synonyms, notably *K. fragilis* and *Saccharomyces keyfr*. Although yeasts in the genus may be isolated from diverse habitats, including

insects and fruit, they are best known because of their association with fermented dairy products, such as cheeses and kefir. The frequent isolation of *K. marxianus* from dairy products, and its long history of safe use, means that this yeast has GRAS (US) and QPS (EU) status (<http://www.efsa.europa.eu/en/efsajournal/pub/3449.htm>). As well as this approval for food use, *K. marxianus* possesses traits that render it attractive for certain biotechnological applications (Fonseca *et al.*, 2008;

Lachance, 2011; Lane and Morrissey, 2010). First is the production of β -galactosidase and inulinase, enzymes that allow the use of lactose and inulin, respectively, as a carbon source. Lactose assimilation is quite rare in yeasts but is possible in *K. marxianus* (and *K. lactis*) because of the presence of the genes *LAC12* and *LAC4*, encoding a lactose permease and a β -galactosidase, respectively. Inulin is a heterogeneous polymer of fructose joined by $\beta(2,1)$ glycosidic bonds and is a common plant storage carbohydrate. The capacity to use inulin (encoded by the *INU1* gene) as a sole carbon source is one of the diagnostic tools used to identify *K. marxianus* strains. The ability of *K. marxianus* to grow on wastes from the cheese (lactose) and plant-processing (inulin) industries is important for the biotechnological applications of this yeast. A second important, industrially relevant trait is thermotolerance, with all *K. marxianus* strains able to grow at 44°C and some able to tolerate growth at temperatures >50°C. High-temperature fermentations reduce cooling costs and also prevent the growth of many contaminating bacteria, most notably coliforms. Third, *K. marxianus* exhibits a remarkably fast growth rate, with generation times as low as 45 min on rich medium (Groeneveld et al., 2009). Fourth, *K. marxianus* is a respiro-fermentative yeast capable of generating energy by either respiration or fermentation. In the latter case, ethanol is a by-product and this has led to various applications in the biofuels sector (Abdel-Banat et al., 2010a; Guimaraes et al., 2010).

***Kluyveromyces marxianus* and flavours in natural fermented products**

Although *K. marxianus* can be isolated from diverse sources, it is best known because of its association with naturally fermented milk products, such as kefir and cheese (Gao et al., 2012). In these cases, the product is produced by the combined activity of a consortium of bacteria and yeasts. These natural products have a wide diversity of flavour compounds, with yeasts likely to play a role in the production of particular esters, ketones, aldehydes and alcohols (Fabre et al., 1995; Plessas et al., 2008). The term 'flavour' is used to describe the full sensory profile of food and beverages,

comprising both smell and taste sensations. Although there are many studies reporting the isolation and identification of *K. marxianus* in fermented milk products, much of what is described is anecdotal and postulated and, in fact, there are few robust data demonstrating the precise role of *K. marxianus* in modulating the flavours of these products. Interestingly, studies that have addressed the flavour contribution of yeasts in mixed fermentations have found that co-culturing yeasts with bacteria seems to enhance flavour production by yeasts, and although the mechanism is not known, it is suggestive of metabolic interactions between the different microbial species growing in a consortium (Martin et al., 2001; Plessas et al., 2008). One recent study of yeast association with flavours in sheep and goat cheese links *K. marxianus* to molecules such as 2-phenylethanol, ethyl decanoate, benzaldehyde and 2,3-butanediol (Padilla et al., 2014). Indeed, while not specific to *Kluyveromyces*, there is a growing recognition of the contribution of non-*Saccharomyces* yeasts to the flavours of fermented beverages (Jolly et al., 2014). Much of what we know about yeast-based production of flavours comes from studies with *Saccharomyces* (Cordente et al., 2012; Pires et al., 2014; Swiegers and Pretorius, 2005), and it is likely that broadly similar metabolic pathways are present in other yeast species. The majority of flavour molecules are derived from central anabolic or catabolic pathways in the cell by the action of modifying enzymes (Figure 1). The same pathways that give rise to desirable flavours are also involved in the synthesis of off-flavours, such as diacetyl and 2,3-butanediol, in some fermented beverages. An important note of caution must be sounded, however, when attempting to extrapolate from *S. cerevisiae* to *K. marxianus*, as the evolutionary history of the two yeasts is very different. First, *S. cerevisiae* underwent a whole-genome duplication c100 MYA that created duplicate copies of all genes (although many have since been lost) (Wolfe and Shields, 1997), and second, optimization of *S. cerevisiae* metabolism for alcoholic fermentation and the subsequent domestication imposed very different selective pressure on the *S. cerevisiae* genome (Dashko et al., 2014; Hagman et al., 2013; Piskur et al., 2006; Scannell et al., 2007). Thus, *S. cerevisiae* is a powerful resource to provide starting points for studying *K. marxianus* genes, proteins, pathways and networks,

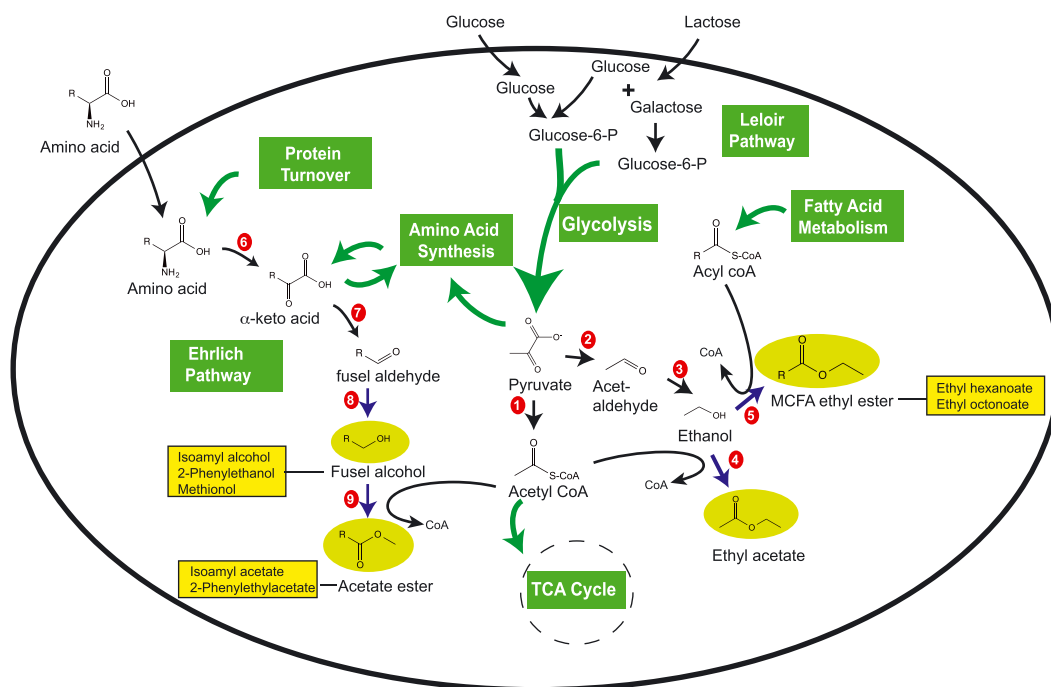


Figure 1. Metabolic pathways for synthesis of aroma and flavour molecules. Molecules with flavour characteristics are derived from normal anabolic and catabolic processes in the cell. Abbreviated versions of how these processes that lead to the formation of classes of metabolite are shown, along with examples cited in the main text. Reactions are depicted as unidirectional, although many are reversible. Important enzymatic steps are indicated by the key, as follows: 1, pyruvate decarboxylase; 2, pyruvate decarboxylase; 3, alcohol dehydrogenase; 4, alcohol acyl transferase; 5, acyl-coenzymeA:ethanol *O*-acyltransferase-keto acid decarboxylase; 6, amino acid transaminase; 7, α -keto-acid decarboxylase; 8, oxidoreductase; 9, alcohol acyl transferase. In *S. cerevisiae*, several of these reactions can be carried out by multiple enzymes, but limited data are available for *K. marxianus*.

but the two yeast species cannot be considered to be equivalent in all aspects. Only a few flavour compounds have been studied in significant detail in *K. marxianus* and these are listed in Table 1 and described below.

Higher alcohols

As well as ethanol, which is derived from the decarboxylation of pyruvate, yeasts synthesize a range of so-called 'higher alcohols' (also described as 'fusel alcohols'). It was first proposed and demonstrated by Ehrlich in 1906 that higher alcohols are synthesized from catabolism of amino acids, and the pathway leading to their formation bears his name (Hazelwood *et al.*, 2008). This (Ehrlich) pathway proceeds in three steps: first, the amino acid is transaminated to create an α -keto acid; next, this α -keto acid is decarboxylated to an aldehyde; and finally, the aldehyde is reduced to an alcohol

or oxidized to a fusel acid, depending on the redox status of the cells (Figure 1, reactions 6, 7 and 8). Higher alcohols may in turn be acted on by alcohol acetyl transferases (AATases) to yield acetate esters (Figure 1, reaction 9). In addition to their synthesis via the Ehrlich pathway, α -keto acids are intermediates in the biosynthesis of certain amino acids and therefore constitute a link between anabolic and catabolic processes. Depending on metabolic fluxes, unbalanced amino acid synthesis can also contribute to synthesis of flavour molecules. Many of the enzymes involved in amino acid metabolism have been identified in *S. cerevisiae*, but there is little detail available from other yeasts. Various branched-chain and aromatic amino acids can serve as the starting point for synthesis of different volatile molecules, but only 2-phenylethanol (2-PE) has been the subject of detailed study in *K. marxianus*. 2-PE is widely used in the flavour industry because of its pleasant rose-like aroma and sweet taste. It is also an

Table 1. *Kluyveromyces* spp. strains used as cell factories for the bioproduction of 2-phenylethanol, 2-phenylethyl acetate and ethyl acetate

Flavour compound	Flavour note	Strain ^a	Production	Productivity ^b (mg/l/h)	Other information	Reference
2-Phenylethanol (2-PE)	Rose	CBS 600	10200 mg/l in 30 h	330	Fed-batch and ISPR ^c w. PPG 1200	Etschmann and Schrader, 2006
		CBS 600	20380 mg/l in 50 h	408	Semi-continuous reactor w. ISPR	Gao and Daugulis, 2009
		CBS 600	5600 mg/l in 39 h	144	With ISPR and optimized medium	Etschmann et al., 2004
		CBS 600	3470 mg/l in 15 h	231	With ISPR by pervaporation	Etschmann et al., 2005
		CBS 397	3050 mg/l in 67 h	45	Bioproduction in molasses w. ISPR	Etschmann et al., 2003
		<i>K. lactis</i> DSMZ 70793	1270 mg/l in 33 h	38	Bioproduction on grape must of Riesling	Etschmann et al., 2003
		CBS 6556	770 mg/l in 76 h	10	Cultivation on grape must of Riesling	Garavaglia et al., 2007
			850 mg/l in 24 h	35	Without ISPR	Fabre et al., 1998
			400 mg/l in 120 h	3	Fed-batch w. glucose as carbon source (<i>de novo</i> biosynthesis)	Fabre et al., 1995
			1300 mg/l in 72 h	18	Evolved <i>K. marxianus</i> overexpressing <i>aroG^{dir}</i> from <i>Klebsiella pneumoniae</i>	Kim et al., 2014b
2-Phenylethyl acetate (2-PEA)	Rose, fruit-like	<i>K. marxianus</i> ^d	1000 mg/l in 72 h	14	<i>K. marxianus</i> overexpressing ARO10 and ADH2 genes from <i>S. cerevisiae</i>	Kim et al., 2014b
		CBS 600	2300 mg/l in 30 h	80	Fed-batch and ISPR w. PPG 1200	Etschmann and Schrader, 2006
		CBS 600	4000 mg/l in 17 h	235	With ISPR by pervaporation	Etschmann et al., 2005
		KY3	435 mg/l in 192 h	2	Higher 2PEA accumulation than <i>K. marxianus</i> CBS600 and CBS6432	Chang et al., 2014
		DSM 5422	17960 mg/l in 18 h	998	Medium produced from whey permeate (<i>de novo</i> biosynthesis) RY ^e = 46%, 0.24 g EA/g lactose	Loser et al., 2013
Ethyl acetate	Fruit	DSM 5422	21470 mg/l in 36 h	596	Media produced from whey permeate (<i>de novo</i> biosynthesis) RY ^e = 56%, 0.29 g EA/g lactose	Urit et al., 2013a
		<i>K. marxianus</i>		700	RY ^e = 27%, 0.14 g EA/g lactose Continuous culture on diluted whey permeate supplemented with ethanol	Kallel-Mhiri and Miclo, 1993

^aUnless specified, all strains are *K. marxianus*.^bOverall productivity = mass produced/unit reaction volume/unit time).^cISPR, *in situ* product removal.^dGenetically engineered yeast strain.^eRY, relative yield (= absolute yield/maximum theoretical yield for the substrate under consideration). RY values are from Loser et al. (2014).

important 'fragrance', a term which is used to describe a single volatile compound with commercial use as an odorant, for example in the perfume industry. Many microbially produced volatiles can be used as both flavour and fragrance compounds. Naturally, 2-PE can be extracted from rose petals, but the bulk of commercial production is via chemical synthesis. It is also synthesized by a range of bacteria and yeasts and these have been identified as possible sources of natural 2-PE for the food industry (Etschmann *et al.*, 2002). It has been known for some time that some strains of *Kluyveromyces* are quite good producers of 2-PE, and a number of studies have addressed the physiological basis of 2-PE synthesis by *K. marxianus*. In addition, optimization strategies have been employed to enhance production with a view to developing commercial production of 2-PE in this yeast (Table 1). The first systematic efforts focused on screening *K. marxianus* strains to identify those with good potential for 2-PE production (Etschmann *et al.*, 2003; Fabre *et al.*, 1995), and on then exploring how nutrition affected levels of 2-PE in the cell (Etschmann *et al.*, 2004; Fabre *et al.*, 1998). Detailed analysis of physiology and metabolism established the pathways for 2-PE production in *K. marxianus*. 2-PE is derived from phenylpyruvate via the catabolism of phenylalanine (Phe) in the Ehrlich pathway (Etschmann *et al.*, 2002). 2-PE can be further metabolized to 2-phenylethylacetate (2-PEA) by a trans-esterification reaction. Natural levels of 2-PE are quite low because of balanced metabolism in the yeast cell, but addition of exogenous L-phenylalanine (L-Phe) to the growth medium, especially as a sole nitrogen source, leads to greatly increased levels of 2-PE (Etschmann *et al.*, 2002; Etschmann *et al.*, 2005; Fabre *et al.*, 1998). A detailed study of L-Phe metabolism and fluxes using ^{13}C labelling found that just over 73% was metabolized via the Ehrlich pathway, with the remainder metabolized either via the cinnamate pathway (22%) or used for biomass generation (4%) (Wittmann *et al.*, 2002). Although it is possible to achieve high conversion of L-Phe to 2-PE, overall yield is limited by the toxicity of 2-PE to *K. marxianus*. The development of strategies for *in situ* product removal (ISPR) of 2-PE during the fermentation has facilitated significantly higher production levels. Thus, although 2-PE is toxic to *K. marxianus* at 2 g/l, it was possible to achieve final overall concentrations of >10 g/l and 20 g/l using liquid-liquid

(Etschmann and Schrader, 2006) or solid-phase (Gao and Daugulis, 2009) ISPR methods, respectively. Summarizing the progress with *K. marxianus*, it can be seen that the maximum overall concentration of 2-PE increased from 400 mg/l to 20.4 g/l and the productivity from 3 mg/l/h to 408 mg/l/h over the course of 14 years of research and development. These gains were largely achieved through process-engineering strategies (see Table 1). As mentioned previously, other yeast species have also been studied for the bioproduction of natural 2-PE, namely *S. cerevisiae*, *Clavispora lusitanae*, *Yarrowia lipolytica*, *Kloeckera saturnus*, *Hansenula anomala*, *Pichia anomala*, *P. membranaefaciens*, *Pichia fermentans* and *Z. rouxii* (Table 2). Among them, the yeast *S. cerevisiae* R-UV3 achieved a higher productivity than the best *K. marxianus* strain CBS 600 (900 vs 408 mg/l/h). The *S. cerevisiae* process was a continuous culture system, whereas the *K. marxianus* system was fed-batch, but in both cases ongoing product removal was the key important feature. This is an area where further developments are likely. For example, adsorption of 2-PE onto polymeric resins has been investigated with good results with *S. cerevisiae* (Carpiné *et al.*, 2013; Hua *et al.*, 2010; Mei *et al.*, 2009). The results can probably be transferred easily to *K. marxianus*, provided there is biocompatibility of the adsorber material. Future process optimization and new reactor concepts (Mihalj *et al.*, 2012a, 2012b, 2013), as well as strain selection or engineering, are likely to further enhance the prospects for commercial production of 2-PE in *K. marxianus*.

Acetate esters

Acetate esters are very significant contributors to flavours in all yeast-based fermented foods and beverages, most notably beer and wine (Pires *et al.*, 2014). Their formation involves the transfer of an acetate group from acetyl-CoA to the hydroxyl group of an alcohol to create the particular acetate ester, depending on the substrate alcohol (Figure 1, reactions 4 and 9). The enzyme catalysing this reaction is an alcohol acetyltransferase (AATase), PFAM PF07247.7, with the best studied being the two paralogous genes in *S. cerevisiae*, *scATF1* and *scATF2* (Verstrepen *et al.*, 2003). Genetic analysis in *S. cerevisiae* indicates that these genes are

Table 2. Other yeast species used as whole cell biocatalysts for the bioproduction of 2-phenylethanol and ethyl acetate

Flavour compound	Flavour note	Yeast	Production	Productivity ^a (mg/l/h)	Other information ^b	Reference	
2-Phenylethanol	Rose	<i>Saccharomyces cerevisiae</i> R-UV3	56700 mg/l in 63 h	900	Fed-batch and continuous ISPR ^c process	Wang et al., 2011	
		<i>Saccharomyces cerevisiae</i> Giv 2009	24000 mg/l in 93 h	258	Concentration in the organic phase	Stark et al., 2002	
		<i>Saccharomyces cerevisiae</i> P-3	6600 mg/l in 39 h	169	ISPR w. adsorption resin	Hua et al., 2010	
		<i>Saccharomyces cerevisiae</i> BD	6170 mg/l in 24 h	257	ISPR w. adsorption resin	Mei et al., 2009	
		<i>Saccharomyces cerevisiae</i> ^d	6100 mg/l in 72 h	85	<i>ald3D ARO80 ARO9 ARO10</i>	Kim et al., 2014a	
		<i>Clavispora lusitanae</i> DSMZ 70102		81	Bioproduction in molasses w. ISPR	Etschmann et al., 2003	
		<i>Saccharomyces cerevisiae</i> Giv 2009	2350 mg/l in 48 h	49		Stark, 2001	
		<i>Yarrowia lipolytica</i> NCYC3825	1980 mg/l in 95 h	21		Celinska et al., 2013	
		<i>Kloeckera saturnus</i> CBS 5761	1700 mg/l in 24 h	71		Albertazzi et al., 1994	
		<i>Hansenula anomala</i> CBS 110	1700 mg/l in 24 h	71		Albertazzi et al., 1994	
		<i>Saccharomyces cerevisiae</i> NCYC 739	1500 mg/l in 24 h	63		Albertazzi et al., 1994	
		<i>Pichia anomala</i> DSMZ 70130	1260 mg/l in 33 h	38	Bioproduction in molasses w. ISPR	Etschmann et al., 2003	
		<i>Pichia membranaefaciens</i> CBS 637	1030 mg/l in 28 h	37	Bioproduction in molasses w. ISPR	Etschmann et al., 2003	
		<i>Saccharomyces cerevisiae</i> DSMZ 70487	950 mg/l in 47 h	20	Bioproduction in molasses w. ISPR	Etschmann et al., 2003	
		<i>Zygosaccharomyces rouxii</i> CBS 5717	1010 mg/l in 33 h	31	Bioproduction in molasses w. ISPR	Etschmann et al., 2003	
		<i>Pichia fermentans</i> L-5	506 mg/l in 16 h	32		Huang et al., 2001	
		<i>Pichia fermentans</i> L-5	453 mg/l in 16 h	28		Huang et al., 2000	
		<i>Saccharomyces cerevisiae</i> NCYC 739	250 mg/l in 28 h	9	Bioproduction in molasses w. ISPR	Etschmann et al., 2003	
	Ethyl acetate (EA)	Fruit	<i>Zygosaccharomyces rouxii</i>	152 mg/l			Aoki and Uchida, 1990
			<i>Candida utilis</i>	15000 mg/l			Armstrong and Brown, 1994
		<i>Candida utilis</i> ATCC 9950	2800 mg/l in 24 h	117	RY ^e = 40%, 0.39 g EA/g ethanol	Armstrong et al., 1984	
		<i>Candida utilis</i> NRC 2721			RY = 55%, 0.27 g EA/g glucose	Williams et al., 1988	
		<i>Pichia anomala</i>			RY = 76%, 0.73 g EA/g ethanol	Bol et al., 1987	
		<i>Pichia anomala</i>			RY = 36%, 0.18 g EA/g glucose	Tabachnick and Joslyn, 1953	
		<i>Zygosaccharomyces rouxii</i>			RY = 22%, 0.21 g EA/g ethanol	Yong et al., 1981	
		<i>Lachancea kluyveri</i>			RY = 16%, 0.08 g EA/g glucose	Moller et al., 2002	
		<i>Williopsis saturnus</i>			RY = 4%, 0.02 g EA/g glucose	Davies et al., 1951	

^aOverall productivity = mass produced/unit reaction volume/unit time.

^bUnless specified, production is by bioconversion.

^cISPR, *in situ* product removal.

^dGenetically engineered yeast strain.

^eRY, relative yield (= absolute yield/maximum theoretical yield for the substrate under consideration). RY values are from Löser et al. (2014).

responsible for the formation of the majority of acetate esters in the cell, although in their absence some smaller esters are still synthesized, indicating that unidentified genes also play some role in ester formation. The most important esters produced by *K. marxianus* are 2-phenylethyl acetate (2-PEA), isoamyl acetate and ethyl acetate, synthesized from 2-PE, isoamyl alcohol and ethanol, respectively (Figure 1, reactions 4 and 9). The levels of acetate esters produced under any particular set of growth conditions will be determined by three parameters: availability of the substrate alcohol; acetyl-CoA levels in the cytoplasm; and expression and activity of the alcohol acetyltransferase(s). The conditions that give rise to elevated levels of higher alcohols (discussed above) thus also provide the circumstances in which elevated levels of acetate esters are possible. Expression levels of AATase are certainly important and, in *S. cerevisiae*, variable expression is known to be an important factor in determining the final ester profile following fermentation (Verstrepen *et al.*, 2003). Despite the importance of 2-PEA and isoamyl acetate, there has been little research in *K. marxianus* on production of these metabolites, other than as by-products of higher alcohol synthesis. The highest yields and productivities have been obtained with *K. marxianus* strain CBS 600 in production processes largely the same as for 2-PE synthesis (Table 1). A recent patent describes a new *K. marxianus* strain, KY3, that was reported to accumulate more 2-PEA than strains CBS600 or CBS6432, when cultivated on glucose medium supplemented with 2.4 g/L-Phe and incubated at 30°C under 'slightly anaerobic' conditions (Chang *et al.*, 2014). Insufficient data are available, however, to allow robust comparison of all the strains and their production traits.

In contrast to 2-PEA, there has been some very detailed research focusing on the production of ethyl acetate, driven mainly because, as well as influencing flavour, ethyl acetate is an important industrial solvent. These studies have sought to develop an ethyl acetate production process from whey permeate, using *K. marxianus* as the production organism (Loser *et al.*, 2014). It is important to note that the metabolic origin of ethyl acetate is quite different and this metabolite is not derived from amino acid metabolism (Figure 1, reaction 4). Since ethanol is the substrate for ethyl acetate, conditions (notably oxygen status) that increase ethanol accumulation also favour ethyl acetate

production. Formation of ethyl acetate itself is an aerobic process (Kallel-Mhiri and Miclo, 1993; Urit *et al.*, 2013b) and precise control of oxygenation is required to optimize ethyl acetate production. Interestingly, however, the regulation of its biosynthesis is more complex and studies have also found that nutrition, growth rates and, especially, activity of the TCA cycle have a profound effect on formation of this ester. The most significant results showed that limiting the metals iron (Fe) and/or copper (Cu) enhanced the production of ethyl acetate (Loser *et al.*, 2012). The hypothesis is that certain enzymes in the TCA cycle are dependent on Fe (aconitase, succinate dehydrogenase) or Cu (electron transport, complex IV) and limiting these elements impairs the TCA cycle. In addition, Fe is also needed for the recycling of NADH to NAD⁺ in the mitochondrion. This dual effect of Fe limitation is postulated to reduce the flux of acetyl CoA into the TCA cycle and thereby increase its availability to the AATase enzyme(s) (Loser *et al.*, 2012). Although this work focused exclusively on ethyl acetate, the conclusions should be directly transferable to other esters that are synthesized by the same enzyme, and it may be possible to enhance the production of esters such as isoamyl acetate and 2-PEA by manipulating acetyl CoA levels, for example by restricting TCA activity.

Extrapolating from *S. cerevisiae*, it is postulated that the majority of acetate esters in *K. marxianus* are synthesized by the single *kmATF* gene. Although *kmATF* is clearly orthologous to *scATF2*, it remains to be experimentally demonstrated that it is functionally equivalent to either *scATF1* or *scATF2*. The best data come from heterologous expression studies, where it was found that *K. lactis* Atfp and *S. cerevisiae* Atf2p had a similar AATase activity but, given that this was <10% of the activity of *S. cerevisiae* Atf1p, it is questionable whether this is the true biological activity of the Atf2p enzyme (Van Laere *et al.*, 2008), which, in *S. cerevisiae*, is also involved in sterol metabolism (Tiwari *et al.*, 2007). A similar conclusion was reached in a recent study that compared flavour production in a number of *K. marxianus* strains (Gethins *et al.*, 2014). Some older literature also reports that ethyl acetate may be formed by the reverse activity of esterases (Kallel-Mhiri and Miclo, 1993; Plata *et al.*, 2003), therefore it is important to be aware that other possible biosynthetic

routes may occur. It is also reported that other yeasts, such as *Candida utilis* (syn. *Lindnera jadinii*), *Pichia anomala* (syn. *Wickerhamomyces anomalus*), *Zygosaccharomyces rouxii*, *Lachancea kluyverii* and *Williopsis saturnus* can synthesize ethyl acetate (Table 2), but differing methodologies preclude comprehensive comparison to *K. marxianus* across all production parameters (Loser et al., 2014).

Strategies to develop *K. marxianus* as a natural F&F production platform

Despite the intrinsic potential of *K. marxianus* for commercial flavour and fragrance production, there are relatively few relevant patents using *K. marxianus* for the production of defined F&F compounds. The most common product with two patents is 2-PE (Chang et al., 2014; Etschmann et al., 2011), followed by one of each for the cheese-like flavour methionol (Leslie and Quan, 2007) and the sweetening steviol glycosides (Lehmann et al., 2013). *K. marxianus* is more frequently used in starter cultures for the fermentation of various substrates, such as milk, cocoa fruits and grape juice (Brandsma and Meijer, 2008; De and Camu, 2007; Mahdavi, 2003; Meijer et al., 2014; Preston and Murphy, 2011; Sommer and Nielsen, 2006). It is also used for the production of various enzymes. Due to its background as a dairy yeast, the application of *K. marxianus* for the production of lactase is obvious (Harju et al., 2008; Swaaf et al., 2007), but it can also be used to manufacture epoxide hydrolase (Botes et al., 2006), sulphhydryl oxidase (Maat et al., 1993) or xylanase for use in flavour production. Biomass, which can be conveniently produced with *K. marxianus* because of its high growth rate and broad substrate spectrum (De Boer et al., 1996; Fieldhouse et al., 2009; Lewandowski, 2011), is often further processed to the natural flavour enhancer yeast extract (Monch and Stute, 2002; Oriol and Kaid, 2009; Potman and Wesdorp, 1995).

The main reason why there are not more examples of application in industry is that the levels of volatile metabolites produced by wild-type *K. marxianus* strains under standard culture conditions are insufficient for commercial production, and therefore commercial development needs innovation at multiple levels. Strategies that can be

implemented for the development of *K. marxianus* cell factories for F&F production are summarized in Figure 2. In effect, there are two parallel approaches to developing optimized strains that have high production levels of the desired metabolite: identification and improvement of wild-type strains; or application of genetic engineering to design tailored strains. The former approach relies on non-GM methodology and seeks to exploit the large diversity of *K. marxianus* that can be found in culture collections. Screening these collections to identify natural variants with higher levels of production is the classical approach, and this has been the first step in most of the studies already discussed in this review (Etschmann et al., 2003; Fabre et al., 1995; Loser et al., 2011). Screening

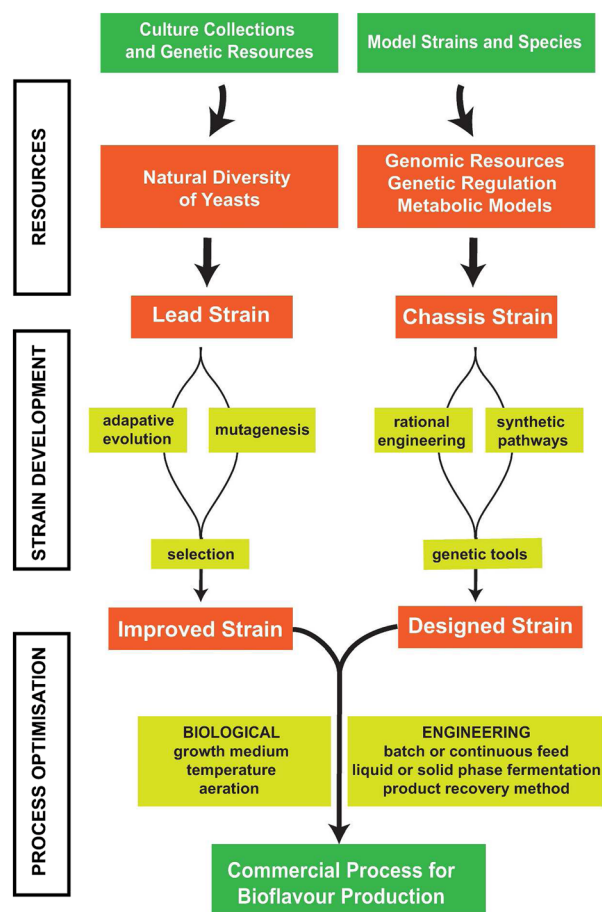


Figure 2. Strategies for developing a commercial bioflavour production process. The strategy can be divided into three phases: accessing of resources; strain development; and process optimization. Alternative 'non-GM' and 'GM' strategies for strain development are presented

is particularly relevant for *K. marxianus*, which appears to be quite a physiologically variable species (Lane *et al.*, 2011; Rocha *et al.*, 2011b). One limitation with this approach is the fact that most cultured *K. marxianus* diversity is found in proprietary strain collections, which limits systematic screening for relevant phenotypes. Once lead strains are identified, they can be further developed through classical mutagenesis and selection or through adaptive evolution and evolutionary engineering, which takes advantage of inherent knowledge of biosynthetic routes and pathways. Although these methods have not yet been implemented with *K. marxianus* for F&F strains, they have been widely applied with industrial strains of *S. cerevisiae* for many different applications. The main challenge with these non-GM methods is identification of the appropriate conditions that will allow the selection of over-producing strains.

The alternative strategy to strain development is to apply molecular methods, both via what is now 'traditional' genetic engineering and using the newer synthetic biology tools that are becoming available. The knowledge gained from studies of model yeasts and strains, and the development of molecular tools, are the key resources that facilitate this designer approach to strain development. An understanding of genes and pathways, of metabolic fluxes and of regulatory steps enables researchers to carry out rational engineering to overcome bottlenecks, remove unnecessary reactions and maximize flux towards the metabolite of interest. There are many examples from *S. cerevisiae*, where these approaches have been successful at modifying flavour profiles (Cordente *et al.*, 2012; Pretorius *et al.*, 2012; Swiegers and Pretorius, 2005). The major restriction to implementation of rational/metabolic engineering in *K. marxianus* has been the lack of sufficient molecular tools, but this limitation is rapidly being overcome. In recent years, several tools have been developed for *K. marxianus* and this is facilitating molecular approaches, both to understand pathways in the yeast and to engineer new ones (Table 3). Some of these tools were recently applied to engineer a *K. marxianus* strain for 2-PE production (Kim *et al.*, 2014b). This study involved overexpressing two genes of the Ehrlich pathway, *ARO10* and *ADH2* (Figure 1, reactions 7 and 8), and a phenylalanine biosynthetic gene,

aroG^{br}. Although the levels of 2-PE production are much lower than in previous studies (Table 1), this work demonstrates the potential of engineering, as levels in the engineered strain were five-fold greater than in the parental strain. It should also be noted that this strain produced 2-PE *de novo* – without the addition of L-Phe to the medium, and so differs from the whole-cell biocatalyst of other studies. Engineering studies have also recently been applied to improved productivity of *S. cerevisiae* by engineering similar steps of the pathway (Kim *et al.*, 2014a) and deletion of the *Saccharomyces cerevisiae* gene *ARO8*, encoding an aromatic amino acid transaminase, also enhances phenylethanol production from glucose (Romagnoli *et al.*, 2014). It is also worth noting the importance of genomics, comparative genomics and comparative physiology. A genome sequence of *K. marxianus* is also available (Jeong *et al.*, 2012) and this will allow the identification of the key genes involved in flavour production. It will be equally important, however, to also compare the physiology and metabolism of *K. marxianus* with other yeasts, as we already know that the species has unique and distinct features. The development of global-scale models of metabolism is likely to contribute significantly to this area. Synthetic biology offers an interesting alternative, as this philosophy centres on the creation of novel pathways by combining genes that encode the requisite enzymatic reactions. Thus, *K. marxianus* could be engineered to produce flavour and fragrance compounds beyond the current spectrum of the capacity of this yeast species. The first synthetic *K. marxianus* cell factory strain was recently described, and the approach that was taken shows the potential of the methodology (Cheon *et al.*, 2014). There have also been recent examples applying synthetic biology to producing F&F compounds in *S. cerevisiae*, notably vanillin (Hayden, 2014) and β -ionone (Beekwilder *et al.*, 2014).

The non-GM vs GM strategies that can be applied mirror developments with *S. cerevisiae*, where the non-GM approach is extensively used by the beverage industry because of regulatory and consumer issues with genetic engineering, whereas the rational/designer approaches are favoured in the pharmaceutical and white biotechnology sectors. There is of course cross-over, as the lessons learned from nature certainly inform

Table 3. Development of molecular tools for engineering *K. marxianus*

Genome sequences		
Genome sequence of the thermotolerant yeast <i>Kluyveromyces marxianus</i> var. <i>marxianus</i> KCTC 17555		Jeong et al., 2012
Genomic exploration of the hemiascomycetous yeasts: 12. <i>Kluyveromyces marxianus</i> var. <i>marxianus</i>		Llorente et al., 2000
Promoters and vectors		
Characterization of <i>Saccharomyces cerevisiae</i> promoters for heterologous gene expression in <i>Kluyveromyces marxianus</i>		Lee et al., 2013
Evaluation of the tetracycline promoter system for regulated gene expression in <i>Kluyveromyces marxianus</i>		Pecota and Da Silva, 2005
Construction of efficient centromeric, multicopy and expression vectors for the yeast <i>Kluyveromyces marxianus</i> using homologous elements and the promoter of a purine-cytosine-like permease		Ball et al., 1999
Markers and genetic integration		
Non-homologous end joining-mediated functional marker selection for DNA cloning in the yeast <i>Kluyveromyces marxianus</i>		Hoshida et al., 2014
Deletion of a KUB80 homolog enhances homologous recombination in the thermotolerant yeast <i>Kluyveromyces marxianus</i>		Choo et al., 2014
Simultaneous integration of multiple genes into the <i>Kluyveromyces marxianus</i> chromosome		Heo et al., 2013
Identification of auxotrophic mutants of the yeast <i>Kluyveromyces marxianus</i> by non-homologous end joining-mediated integrative transformation with genes from <i>Saccharomyces cerevisiae</i>		Yarimizu et al., 2013
Random and targeted gene integrations through the control of non-homologous end joining in the yeast <i>Kluyveromyces marxianus</i>		Abdel-Banat et al., 2010b
Sequential gene integration for the engineering of <i>Kluyveromyces marxianus</i>		Pecota et al., 2007
Application of the Cre-LoxP system for multiple gene disruption in the yeast <i>Kluyveromyces marxianus</i>		Ribeiro et al., 2007
Transformation system for prototrophic industrial yeasts using the <i>AUR1</i> gene as a dominant selection marker		Hashida-Okado et al., 1998
Construction of recombinant strains		
A biosynthetic pathway for hexanoic acid production in <i>Kluyveromyces marxianus</i>		Cheon et al., 2014
Biosynthesis of 2-phenylethanol from glucose with genetically engineered <i>Kluyveromyces marxianus</i>		Kim et al., 2014b
Heterologous expression of a thermophilic esterase in <i>Kluyveromyces marxianus</i> yeasts		Rocha et al., 2011a
Heterologous expression of glucose oxidase in the yeast <i>Kluyveromyces marxianus</i>		Rocha et al., 2011a
Cloning and characterization of <i>Kluyveromyces marxianus</i> <i>HOG1</i> gene		Qian et al., 2010
High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast <i>Kluyveromyces marxianus</i> DMKU3-1042		Nonklang et al., 2008
Construction of thermotolerant yeast expressing thermostable cellulase genes		Hong et al., 2007
Construction of flocculent <i>Kluyveromyces marxianus</i> strains suitable for high-temperature ethanol fermentation		Nonklang et al., 2009
Expression of an α -galactosidase gene under control of the homologous inulinase promoter in <i>Kluyveromyces marxianus</i>		Bergkamp et al., 1993

metabolic engineering, with the term 'inverse engineering' used to describe the process by which traits identified in (experimentally) selected strains are introduced into genetically-clean host strains (Hirosawa *et al.*, 2004; Særens *et al.*, 2010). It is worth noting that the requirement for 'natural' metabolites does not preclude genetic-engineering approaches for strain improvement as, unlike yeasts used in brewing or wine-making, there is no GM material in the final product (the fragrance or flavour compound) and thus there are no labelling requirements. This is analogous to the microbial platforms currently used to produce most vitamins for use in supplements.

The final, and often most challenging, step in cell factory development is taking a high-producing strain and developing a viable production process. Some of the biological and engineering parameters that need to be considered are listed in Figure 2. Effective process optimization is a knowledge-led process where insights gained from fundamental studies on metabolism, gene expression and protein activity are used to guide experimental approaches. This also needs to consider what feedstocks are available, and the capacity of *K. marxianus* to utilize lactose and other sugars is important in this regard. Once growth conditions are established, it becomes a process-engineering challenge, where issues as to type of fermentation, methods for recovering product and life cycle assessment all come into play. Examples of how both biological and engineering approaches have been applied to develop *K. marxianus* for the production of 2-PE and ethyl acetate are described above.

Concluding comments

Across a wide range of cell factory applications, *K. marxianus* is emerging as a genuine competitor to *S. cerevisiae* and, in many cases, may be a superior option. Specifically for flavours and fragrances, the published data indicate that higher yields and productivities can be achieved in *K. marxianus*. There are, however, significant knowledge gaps and much of what has been achieved was through systematic and empirical approaches. To move to the next level – commercial production platforms – it is necessary to integrate the different aspects of strain and process development. It is especially important to fill

the knowledge gaps around genes, pathways, enzymes and regulation, so that we have a comprehensive understanding of how *K. marxianus* produces relevant metabolites. This information can then be used to inform strain development. In the future, the newer methods of strain engineering and synthetic biology will be combined with traditional skills of process development and optimization, in a holistic approach for new cell factory applications of *K. marxianus* in the commercial production of natural flavours and fragrances.

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References

- Abdel-Banat BM, Hoshida H, Ano A, *et al.* 2010a. High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? *Appl Microbiol Biotechnol* **85**: 861–867.
- Abdel-Banat BM, Nonklang S, Hoshida H, *et al.* 2010b. Random and targeted gene integrations through the control of non-homologous end joining in the yeast *Kluyveromyces marxianus*. *Yeast* **27**: 29–39.
- Albertazzi E, Cardillo R, Servi S, *et al.* 1994. Biogenesis of 2-phenylethanol and 2-phenylacetate important aroma components. *Biotechnol Lett* **16**: 491–496.
- Aoki T, Uchida K. 1990. Enhanced formation of 2-phenylethanol in *Zygosaccharomyces rouxii* due to prephenate dehydrogenase deficiency. *Agric Biol Chem* **54**: 273–274.
- Armstrong DW, Brown LA. 1994. *Bioprocess Production of Flavor, Fragrance and Color Ingredients*. Wiley: New York.
- Armstrong DW, Martin SM, Yamazaki H. 1984. Production of ethyl acetate from dilute ethanol solutions by *Candida utilis*. *Biotechnol Bioeng* **26**: 1038–1041.
- Ball MM, Raynal A, Guerneau M, *et al.* 1999. Construction of efficient centromeric, multicopy and expression vectors for the yeast *Kluyveromyces marxianus* using homologous elements and the promoter of a purine-cytosine-like permease. *J Mol Microbiol Biotechnol* **1**: 347–353.
- Beekwilder J, van Rossum HM, Koopman F, *et al.* 2014. Polycistronic expression of a β -carotene biosynthetic pathway in *Saccharomyces cerevisiae* coupled to β -ionone production. *J Biotechnol*. DOI: 10.1016/j.jbiotec.2013.12.016.
- Bergkamp RJ, Bootsman TC, Toschka HY, *et al.* 1993. Expression of an α -galactosidase gene under control of the homologous inulinase promoter in *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **40**: 309–317.
- Bol JJ, Knol W, ten Brik B. 1987. Optimization of the production of ethyl acetate from ethanol by *Hansenula anomala*. *Dechema Monogr* **105**: 235–236.

- Botes AL, Labuschagne M, Lotter J, et al. 2006. Methods for obtaining optically active glycidyl ethers and optically active vicinal diols from racemic substrates. WO/2006/109198A2. World Intellectual Property Organization: Geneva.
- Brandsma JB, Meijer WC. 2008. Use of yeast and bacteria for making dairy products with improved flavour and/or texture quality characteristics. WO/2008/030089A1. World Intellectual Property Organization: Geneva.
- Carpiné D, Dagostin JLA, da Silva VR, et al. 2013. Adsorption of volatile aroma compound 2-phenylethanol from synthetic solution onto granular activated carbon in batch and continuous modes. *J Food Eng* **117**: 370–377.
- Celinska E, Kubiak P, Bialas W, et al. 2013. *Yarrowia lipolytica*: the novel and promising 2-phenylethanol producer. *J Ind Microbiol Biotechnol* **40**: 389–392.
- Chang JJ, Ho CY, Huang CC, et al. 2014. Flavor compound-producing yeast strains. US Patent No. 8703474 B2.
- Cheon Y, Kim JS, Park JB, et al. 2014. A biosynthetic pathway for hexanoic acid production in *Kluyveromyces marxianus*. *J Biotechnol* **182**: 30–36.
- Choo JH, Han C, Kim JY, et al. 2014. Deletion of a *KU80* homolog enhances homologous recombination in the thermotolerant yeast *Kluyveromyces marxianus*. *Biotechnol Lett* **36**: 2059–67.
- Cordente AG, Curtin CD, Varela C, et al. 2012. Flavour-active wine yeasts. *Appl Microbiol Biotechnol* **96**: 601–618.
- Dashko S, Zhou N, Compagno C, et al. 2014. Why, when, and how did yeast evolve alcoholic fermentation? *FEMS Yeast Res* **14**: 826–32.
- Davies R, Falkiner EA, Wilkinson JF, et al. 1951. Ester formation by yeasts. 1. Ethyl acetate formation by *Hansenula* species. *Biochem J* **49**: 58–61.
- De Boer R, Kramer RK, McKernan RW. 1996. Production of a cultured yeast product from whey permeate, yeast cream and yeast centrate. US5486368. Alexandria, Virginia.
- De VL, Camu N. 2007. Starter cultures and fermentation method. WO/2007/031186A1. World Intellectual Property Organization: Geneva.
- Etschmann M, Schrader J, Sell D. 2011. Verfahren zur fermentativen Herstellung von Duft-und/oder Aromastoffen. EP1715032A1. European Patent Office, The Hague.
- Etschmann MMW, Bluemke W, Sell D, et al. 2002. Biotechnological production of 2-phenylethanol. *Appl Microbiol Biotechnol* **59**: 1–8.
- Etschmann MMW, Schrader J. 2006. An aqueous–organic two-phase bioprocess for efficient production of the natural aroma chemicals 2-phenylethanol and 2-phenylethylacetate with yeast. *Appl Microbiol Biotechnol* **71**: 440–443.
- Etschmann MMW, Sell D, Schrader J. 2003. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. *Biotechnol Lett* **25**: 531–536.
- Etschmann MMW, Sell D, Schrader J. 2004. Medium optimization for the production of the aroma compound 2-phenylethanol using a genetic algorithm. *J Mol Catal B Enzymat* **29**: 187–193.
- Etschmann MMW, Sell D, Schrader J. 2005. Production of 2-phenylethanol and 2-phenylethylacetate from L-phenylalanine by coupling whole-cell biocatalysis with organophilic pervaporation. *Biotechnol Bioeng* **92**: 624–634.
- Fabre CE, Blanc PJ, Goma G. 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. *Biotechnol Prog* **14**: 270–274.
- Fabre CE, Duviau VJ, Blanc PJ, et al. 1995. Identification of volatile flavour compounds obtained in culture of *Kluyveromyces marxianus*. *Biotechnol Lett* **17**: 1207–1212.
- Fieldhouse R, MacLennan DF, MacLennan DG. 2009. Process for preparing nutritional, therapeutic or organoleptic products from crude glycerol. WO/2009/089593A1. World Intellectual Property Organization: Geneva.
- Fonseca GG, Heinzle E, Wittmann C, et al. 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl Microbiol Biotechnol* **79**: 339–54.
- Gao F, Daugulis AJ. 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. *Biotechnol Bioeng* **104**: 332–339.
- Gao J, Gu F, Abdella NH, et al. 2012. Investigation on culturable microflora in Tibetan kefir grains from different areas of china. *J Food Sci* **77**: M425–433.
- Gethins L, Guneser O, Demirkol A, et al. 2014. Influence of carbon and nitrogen source on production of volatile fragrance and flavour metabolites by the yeast *Kluyveromyces marxianus*. *Yeast* DOI: 10.1002/yea.3047.
- Groeneveld P, Stouthamer AH, Westerhoff HV. 2009. Super life – how and why ‘cell selection’ leads to the fastest-growing eukaryote. *FEBS J* **276**: 254–270.
- Guimaraes PM, Teixeira JA, Domingues L. 2010. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. *Biotechnol Adv* **28**: 375–384.
- Hagman A, Sall T, Compagno C, et al. 2013. Yeast ‘make–accumulate–consume’ life strategy evolved as a multi-step process that predates the whole genome duplication. *PLoS One* **8**: e68734.
- Harju M, Kajander K, Sibakov T. 2008. Method for producing products containing galacto-oligosaccharides and use thereof. WO/2008/037839A1. World Intellectual Property Organization: Geneva.
- Hashida-Okado T, Ogawa A, Kato I, et al. 1998. Transformation system for prototrophic industrial yeasts using the *AURI* gene as a dominant selection marker. *FEBS Lett* **425**: 117–122.
- Hayden EC. 2014. Synthetic-biology firms shift focus. *Nature* **505**: 598.
- Hazelwood LA, Daran JM, van Maris AJ, et al. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* **74**: 2259–2266.
- Heo P, Yang TJ, Chung SC, et al. 2013. Simultaneous integration of multiple genes into the *Kluyveromyces marxianus* chromosome. *J Biotechnol* **167**: 323–325.
- Hirosawa I, Aritomi K, Hoshida H, et al. 2004. Construction of a self-cloning sake yeast that overexpresses alcohol acetyltransferase gene by a two-step gene replacement protocol. *Appl Microbiol Biotechnol* **65**: 68–73.
- Hong J, Wang Y, Kumagai H, et al. 2007. Construction of thermotolerant yeast expressing thermostable cellulase genes. *J Biotechnol* **130**: 114–123.
- Hoshida H, Murakami N, Suzuki A, et al. 2014. Non-homologous end joining-mediated functional marker selection for DNA cloning in the yeast *Kluyveromyces marxianus*. *Yeast* **31**: 29–46.
- Hua D, Lin S, Li Y, et al. 2010. Enhanced 2-phenylethanol production from L-phenylalanine via *in situ* product adsorption. *Biocatal Biotransfor* **28**: 259–266.
- Huang CJ, Lee CJ, Chou CC. 2000. Production and molar yield of 2-phenylethanol by *Pichia fermentans* L-5 as affected by some medium components. *J Biosci Bioeng* **90**: 142–147.
- Huang CJ, Lee CJ, Chou CC. 2001. Production of 2-phenylethanol, a flavor ingredient, by *Pichia fermentans* L-5 under various culture conditions. *Food Res Int* **34**: 277–282.

- Jeong H, Lee DH, Kim SH, *et al.* 2012. Genome sequence of the thermotolerant yeast *Kluyveromyces marxianus* var. *marxianus* KCTC 17555. *Eukaryot Cell* **11**: 1584–1585.
- Jolly NP, Varela C, Pretorius IS. 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res* **14**: 215–237.
- Kallel-Mhiri H, Miclo A. 1993. Mechanism of ethyl acetate synthesis by *Kluyveromyces fragilis*. *FEMS Microbiol Lett* **111**: 207–212.
- Kim B, Cho BR, Hahn JS. 2014a. Metabolic engineering of *Saccharomyces cerevisiae* for the production of 2-phenylethanol via Ehrlich pathway. *Biotechnol Bioeng* **111**: 115–124.
- Kim TY, Lee SW, Oh MK. 2014b. Biosynthesis of 2-phenylethanol from glucose with genetically engineered *Kluyveromyces marxianus*. *Enzyme Microb Technol* **61–62**: 44–47.
- Kurtzman CP. 2003. Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the Saccharomycetaceae, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygorulasporea*. *FEMS Yeast Res* **4**: 233–245.
- Lachance MA. 2007. Current status of *Kluyveromyces* systematics. *FEMS Yeast Res* **7**: 642–645.
- Lachance MA. 2011. *Kluyveromyces* van der Walt (1971). In *Yeasts: A Taxonomic Study*, 5th edn, chapter 2. Elsevier, Amsterdam: 471–481.
- Lane MM, Burke N, Karreman R, *et al.* 2011. Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. *Antonie Van Leeuwenhoek* **100**: 507–519.
- Lane MM, Morrissey JP. 2010. *Kluyveromyces marxianus*: a yeast emerging from its sister's shadow. *Fungal Biol Rev* **24**: 17–26.
- Lee KS, Kim JS, Heo P, *et al.* 2013. Characterization of *Saccharomyces cerevisiae* promoters for heterologous gene expression in *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **97**: 2029–2041.
- Lehmann M, Trueheart J, Zwartjens P, *et al.* 2013. Diterpene production. WO/2013/110673A1. World Intellectual Property Organization: Geneva.
- Leslie CV, Quan LS. 2007. Dairy product and process. WO/2007/136280A1. World Intellectual Property Organization: Geneva.
- Lewandowski R. 2011. Fermentation process of a substrate using a mixed culture for the production of an edible biomass for animal and/or human consumption. WO/2011/140649A1. World Intellectual Property Organization: Geneva.
- Llorente B, Malpertuy A, Blandin G, *et al.* 2000. Genomic exploration of the hemiascomycetous yeasts: 12. *Kluyveromyces marxianus* var. *marxianus*. *FEBS Lett* **487**: 71–75.
- Loser C, Urit T, Bley T. 2014. Perspectives for the biotechnological production of ethyl acetate by yeasts. *Appl Microbiol Biotechnol* **98**: 5397–5415.
- Loser C, Urit T, Forster S, *et al.* 2012. Formation of ethyl acetate by *Kluyveromyces marxianus* on whey during aerobic batch and chemostat cultivation at iron limitation. *Appl Microbiol Biotechnol* **96**: 685–696.
- Loser C, Urit T, Nehl F, *et al.* 2011. Screening of *Kluyveromyces* strains for the production of ethyl acetate: design and evaluation of a cultivation system. *Eng Life Sci* **11**: 369–381.
- Loser C, Urit T, Stukert A, *et al.* 2013. Formation of ethyl acetate from whey by *Kluyveromyces marxianus* on a pilot scale. *J Biotechnol* **163**: 17–23.
- Maat JURVL, Musters WURVL, Stam HURVL, *et al.* 1993. Cloning and expression of DNA encoding a ripening form of a polypeptide having sulfhydryl oxidase activity. EP0565172A1. European Patent Office, The Hague.
- Mahdavi J. 2003. Multicultural fermented yogurt. WO/2003/090546A1. World Intellectual Property Organization: Geneva.
- Martin N, Berger C, Le Du C, *et al.* 2001. Aroma compound production in cheese curd by coculturing with selected yeast and bacteria. *J Dairy Sci* **84**: 2125–35.
- Mei J, Min H, Lü Z. 2009. Enhanced biotransformation of L-phenylalanine to 2-phenylethanol using an *in situ* product adsorption technique. *Process Biochem* **44**: 886–890.
- Meijer WC, Brandsma JB, Hafkamp AAG. 2014. Cheese having sheep-like and/or goatly flavour attributes. WO/2014/038946A2. World Intellectual Property Organization, Geneva.
- Mihaš M, Krištofiková Ľ, Markoš J. 2013. Production of 2-phenylethanol in hybrid system using airlift reactor and immersed hollow fiber membrane module. *Chem Eng Process* **72**: 144–152.
- Mihaš M, Vereš R, Markoš J, *et al.* 2012a. Intensification of 2-phenylethanol production in fed-batch hybrid bioreactor: biotransformations and simulations. *Chem Eng Process* **57–58**: 75–85.
- Mihaš M, Vereš R, Markoš J. 2012b. Investigation of 2-phenylethanol production in fed-batch hybrid bioreactor: membrane extraction and microfiltration. *Sep Purif Technol* **95**: 126–135.
- Moller K, Christensen B, Forster J, *et al.* 2002. Aerobic glucose metabolism of *Saccharomyces kluyveri*: growth, metabolite production, and quantification of metabolic fluxes. *Biotechnol Bioeng* **77**: 186–193.
- Monch S, Stute R. 2002. Process for producing yeast extracts. US Patent No. 20020081357.
- Nonklang S, Abdel-Banat BM, Cha-aim K, *et al.* 2008. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3–1042. *Appl Environ Microbiol* **74**: 7514–7521.
- Nonklang S, Ano A, Abdel-Banat BM, *et al.* 2009. Construction of flocculent *Kluyveromyces marxianus* strains suitable for high-temperature ethanol fermentation. *Biosci Biotechnol Biochem* **73**: 1090–1095.
- Oriol E, Kaid N. 2009. Novel preparation of phosphodiesterase of plant origin. US Patent No. 20090324778.
- Padilla B, Belloch C, López-Díez JJ, *et al.* 2014. Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses. *Intl Dairy J* **35**: 122–129.
- Pecota DC and Da Silva NA. 2005. Evaluation of the tetracycline promoter system for regulated gene expression in *Kluyveromyces marxianus*. *Biotechnol Bioeng* **92**: 117–123.
- Pecota DC, Rajgarhia V, Da Silva NA. 2007. Sequential gene integration for the engineering of *Kluyveromyces marxianus*. *J Biotechnol* **127**: 408–416.
- Pires EJ, Teixeira JA, Branyik T, *et al.* 2014. Yeast: the soul of beer's aroma – a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl Microbiol Biotechnol* **98**: 1937–1949.
- Piskur J, Rozpedowska E, Polakova S, *et al.* 2006. How did *Saccharomyces* evolve to become a good brewer? *Trends Genet* **22**: 183–186.
- Plata C, Millán C, Mauricio JC, *et al.* 2003. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiol* **20**: 217–224.
- Plessas S, Bosnea L, Psarianos C, *et al.* 2008. Lactic acid production by mixed cultures of *Kluyveromyces marxianus*. *Bioresour Technol* **99**: 5951–5955.

- Potman RP, Wesdorp JJ. 1995. Verfahren zur Herstellung von Hefeextrakt, Hefeextrakt, seine Verwendung als Würzstoff und Nahrungsmittel, das den Hefeextrakt enthält. EP0354610A1. European Patent Office, The Hague.
- Preston D, Murphy RB. 2011. Epicatechin compositions and methods. US Patent No. 20110280933.
- Pretorius IS, Curtin CD, Chambers PJ. 2012. The winemaker's bug: from ancient wisdom to opening new vistas with frontier yeast science. *Bioeng Bugs* **3**: 147–156.
- Qian J, Qin X, Yin Q, et al. 2010. Cloning and characterization of *Kluyveromyces marxianus* HOG1 gene. *Biotechnol Lett* **33**: 571–575.
- Ribeiro O, Gombert AK, Teixeira JA, et al. 2007. Application of the Cre–LoxP system for multiple gene disruption in the yeast *Kluyveromyces marxianus*. *J Biotechnol* **131**: 20–26.
- Rocha SN, Abrahao-Neto J, Cerdan ME, et al. 2011a. Heterologous expression of a thermophilic esterase in *Kluyveromyces* yeasts. *Appl Microbiol Biotechnol* **89**: 375–385.
- Rocha SN, Abrahao-Neto J, Gombert AK. 2011b. Physiological diversity within the *Kluyveromyces marxianus* species [corrected]. *Antonie Van Leeuwenhoek* **100**: 619–630.
- Romagnoli G, Knijnenburg TA, Liti G, et al. 2014. Deletion of the *Saccharomyces cerevisiae* ARO8 gene, encoding an aromatic amino acid transaminase, enhances phenylethanol production from glucose. *Yeast* DOI: 10.1002/yea.3015.
- Saerens SM, Duong CT and Nevoigt E. 2010. Genetic improvement of brewer's yeast: current state, perspectives and limits. *Appl Microbiol Biotechnol* **86**: 1195–1212.
- Scannell DR, Butler G, Wolfe KH. 2007. Yeast genome evolution – the origin of the species. *Yeast* **24**: 929–942.
- Sommer P, Nielsen J. 2006. Yeast compositions and starter cultures. US Patent No. 20060257529.
- Stark D 2001. Extractive bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. In PhD thesis, University of Lausanne, Lausanne.
- Stark D, Munch T, Sonnleitner B, et al. 2002. Extractive bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. *Biotechnol Prog* **18**: 514–523.
- Swaaf DMPM, Dijk VAA, Edens L, et al. 2007. Enzyme preparations yielding a clean taste. WO/2007/060247A2. World Intellectual Property Organization: Geneva.
- Swiegers JH, Pretorius IS. 2005. Yeast modulation of wine flavor. *Adv Appl Microbiol* **57**: 131–175.
- Tabachnick J, Joslyn MA. 1953. Formation of esters of yeast. I. The production of ethyl acetate by standing surface cultures of *Hansenula anomala*. *J Bacteriol* **65**: 1–9.
- Tiwari R, Koffel R, Schneider R. 2007. An acetylation/deacetylation cycle controls the export of sterols and steroids from *S. cerevisiae*. *EMBO J* **26**: 5109–5119.
- Urit T, Li M, Bley T, et al. 2013a. Growth of *Kluyveromyces marxianus* and formation of ethyl acetate depending on temperature. *Appl Microbiol Biotechnol* **97**: 10359–10371.
- Urit T, Manthey R, Bley T, et al. 2013b. Formation of ethyl acetate by *Kluyveromyces marxianus* on whey: influence of aeration and inhibition of yeast growth by ethyl acetate. *Eng Life Sci* **13**: 247–260.
- Van Laere SD, Saerens SM, Verstrepen KJ, et al. 2008. Flavour formation in fungi: characterisation of KIAf, the *Kluyveromyces lactis* orthologue of the *Saccharomyces cerevisiae* alcohol acetyltransferases Atf1 and Atf2. *Appl Microbiol Biotechnol* **78**: 783–792.
- Verstrepen KJ, Derdelinckx G, Dufour JP, et al. 2003. The *Saccharomyces cerevisiae* alcohol acetyl transferase gene *ATF1* is a target of the cAMP/PKA and FGM nutrient-signalling pathways. *FEMS Yeast Res* **4**: 285–296.
- Wang H, Dong Q, Meng C, et al. 2011. A continuous and adsorptive bioprocess for efficient production of the natural aroma chemical 2-phenylethanol with yeast. *Enzyme Microb Technol* **48**: 404–407.
- Williams RE, Armstrong DW, Murray WD, et al. 1988. Enzyme and whole cell production of flavor and fragrance compounds. *Ann NY Acad Sci* **542**: 406–412.
- Wittmann C, Hans M, Bluemke W. 2002. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. *Yeast* **19**: 1351–1363.
- Wolfe KH, Shields DC. 1997. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387**: 708–713.
- Yarimizu T, Nonklang S, Nakamura J, et al. 2013. Identification of auxotrophic mutants of the yeast *Kluyveromyces marxianus* by non-homologous end joining-mediated integrative transformation with genes from *Saccharomyces cerevisiae*. *Yeast* **30**: 485–500.
- Yong FM, Lee KH, Wong HA. 1981. The production of ethyl acetate by soy yeast *Saccharomyces rouxii* NRRL Y-1096. *J Food Technol* **16**: 177–184.