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Review

A review of natural astaxanthin production in a circular bioeconomy context using *Paracoccus carotinifaciens*

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HIGHLIGHTS

- Microbial-based AXT reveals high biological activity than synthetic.
- Bacterial sources produce more natural AXT than yeast and microalgae.
- The thin cell wall of *Paracoccus* contribute to the reduction of production costs.
- *Paracoccus*-based AXT have potential for application of food ingredients.

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ABSTRACT

Astaxanthin (AXT) is a ketocarotenoid with several properties, including antioxidant, antidiabetic and anticancer with a wide range of applications in cosmeceutical, feed, food and pharmaceuticals sectors. The large fraction of AXT available in the market is obtained by chemical route, but the consumers preference for natural products are changing the global market of AXT, and due to that several companies are looking for potential alternative sources such as Gram-negative bacteria *Paracoccus carotinifaciens* (*P. carotinifaciens*) to obtain natural AXT. The aim of this critical review is to provide a comprehensive overview of the latest AXT research findings and characteristics of the hyperproducer-AXT *P. carotinifaciens*. Moreover, a brief description of the potential application of *P. carotinifaciens* for the production of natural AXT at industrial scale for commercial purposes and the latest advancements in the upstream and downstream procedures following the biorefinery and circular economy percepts are considered.

1. Introduction

Astaxanthin (AXT), is a dark-red ketocarotenoid that belongs to the xanthophylls group, widely used in the animal feed, food, cosmeceuticals and pharmaceuticals applications due to the attractive anti-inflammatory, anti-oxidant and anti-proliferative biological properties (Chen et al., 2022; Kohandel et al., 2022; Patel et al., 2022). In recent years, due to the numerous health benefits of this outstanding molecule, the industrial and research interest in AXT has increased exponentially, as can be seen by the bibliometric analysis conducted by us in *Scopus database* (using the term 'astaxanthin' or 'natural astaxanthin') (Fig. 1). From 1960 to 2021 more than 6725 documents were published concerning the all types of AXT (synthetic or natural), from them approximately 28 % are related to natural-based AXT (Fig. 1A). The results

revealed that the interest of the scientific community regarding the AXT has increased in the last decade, viz., from 170 publications in 2010 to almost 712 in 2021. In the same period, only 27 documents were published in 2010 regarding the natural-based AXT, compared to the 201 publications in 2021. This significant increase in the interest for natural-based AXT are directly related to the consumers preference for natural products (Khoo et al., 2019; Mussagy et al., 2021a). Recently the European Food Safety Authority (EFSA) concludes that: 'the intake of 8 mg/day of natural-AXT from food supplements is safe for adults even in combination with an excessive exposure from the background diet' (Turck et al., 2020). Microalgae-based AXT approved by Food and Drug Administration (FDA) has also attracted much attention in recent years due to its highest antioxidant ability compared to the synthetic ones (Mussagy et al., 2021). China (1407), USA (816) and Japan (777) ranks as the

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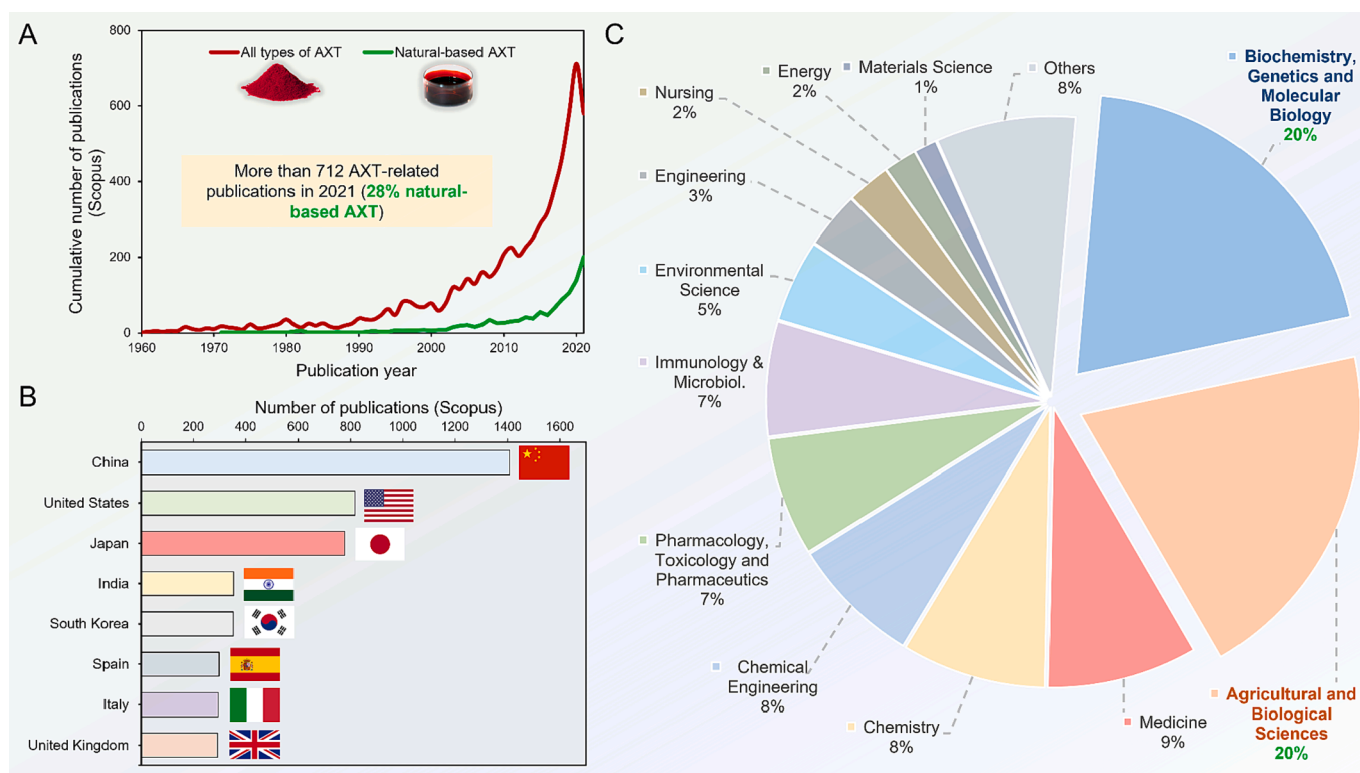


Fig. 1. A Astaxanthin cumulative number of publications *per year*; B contributions of most significant countries; and C some important fields of papers published from 1960 to 2021 (Scopus database). Note that, ‘*all type of AXT*’ a referred to the synthetic, microbial, plant and marine-based astaxanthin.

countries with the largest number of documents published from 1960 to 2021 (Fig. 1B). The growth in publications of AXT in these 3 countries is a direct result of the increasing number of new companies such as: Cyanotech Corp. (USA), FENCHEM (China) and ENEOS corporation (Japan) and new findings on the positive role of AXT in several subjects including biochemistry, genetics and molecular biology (20%), agricultural and biological sciences (20%) and medicine (9%) (Fig. 1C). Note that, the use of ‘*health and natural-based claims*’ by several companies is part of marketing plan to accelerate the regulatory approvals for their growing products lines and open new opportunities for the international market.

The most popular producers of AXT are microalgae (*Haematococcus pluvialis* and *Chlorella zofingiensis*) (Niizawa et al., 2021; Yang et al., 2021), yeast (*Phaffia rhodozyma* and *Rhodospiridium* spp.) (Mussagy et al., 2021a; Mussagy et al., 2022c) and bacteria (*Paracoccus* spp., *Agrobacterium* spp., *Sphingomonas* spp., *Pseudomonas* spp.) (Lien et al., 2018). Among these, the most encouraging systems that produce highest yields of AXT with a certainly application in biorefineries are *Haematococcus pluvialis* 2.7 mg/L/day, *Paracoccus carotinifaciens* (~863 mg/L) and *Phaffia rhodozyma* (~5.2 mg/L) (Hirasawa et al., 2019, 2017; Orosa et al., 2001; Parajó et al., 1998). Alongside the advantages, viz., high biomass production, low nutritional requirements for the intracellular accumulation of AXT and its extensive safety evaluation has revealed significant differences between *Haematococcus pluvialis* and other microbial sources (yeast and bacteria) (Mussagy et al., 2022a), resulting in a clear preference for this as natural cell factory by industries. Therefore, the use of yeast (*P. rhodozyma*) as biological system for producing AXT has been appearing as an interesting source alternative to produce AXT at industrial scale, due to the ability to convert low-cost carbon sources into AXT as well as reduced cultivation time (Mussagy et al., 2022c). However, the presence of a thick cell wall that needs additional high-cost processing steps, such as sequentially cell-disruption and purification procedures (if necessary) for the efficient fractionation and recovery of AXT are the main drawback of these microbial sources (Mussagy

et al., 2021a).

Recently, some findings have cogitated the potential production of natural AXT using wild type or genetically modified *Paracoccus carotinifaciens* (*P. carotinifaciens*) (Hayashi et al., 2021). These results demonstrated that significant improvements in the cultivation of this bacteria are still required to improve the production of AXT at industrial scale. However, compared with the yeast source, the aerobic Gram-negative bacterium *P. carotinifaciens* has a thin cell wall that facilitates the recovery of intracellular AXT, which is an advantage for the industrial application due to the reduction cost of downstream procedures.

Following these encouraging reports, in the present review, the upstream and downstream processing technologies to obtain *P. carotinifaciens*-based AXT are described, starting from taxonomy and characteristics of the genus, AXT biosynthesis, characteristics of produced AXT, improvements in the production to the biomass treatment, cell-disruption, recovery and purification of AXT envisioning the implementation of this microorganism in a biorefinery process following the concept of circular bioeconomy.

2. Taxonomy and characteristics of genus *Paracoccus*

The genus *Paracoccus* is an aerobic Gram-negative, urease-negative, and oxidase/catalase-positive bacterium that revealed a significant metabolic versatility (Zhao et al., 2022). Phylogenetically, the genus *Paracoccus* belongs to the phylum Proteobacteria, class Alphaproteobacteria, order Rhodobacterales, family Rhodobacteraceae, that contain more than 96 recognized species from 1969 to 2022 (Cui et al., 2022; Davis et al., 1969). The name *Paracoccus* derives from the Greek ‘*Para*’ (alongside of, resembling) and Latin ‘*coccus*’ (grain, berry) (Davis et al., 1969). The most representative carotenogenic species from the genera are *P. carotinifaciens*, *P. marcusii* and *P. haeundaensis* (Conradie et al., 2018; Hayashi et al., 2021). The name of the specie ‘*carotinifaciens*’ originates from the Latin ‘*carotinum*’ (carotene); ‘*faciens*’ (making/producing), viz., *carotinifaciens* (carotenoid-producing) (Tsubokura et al.,

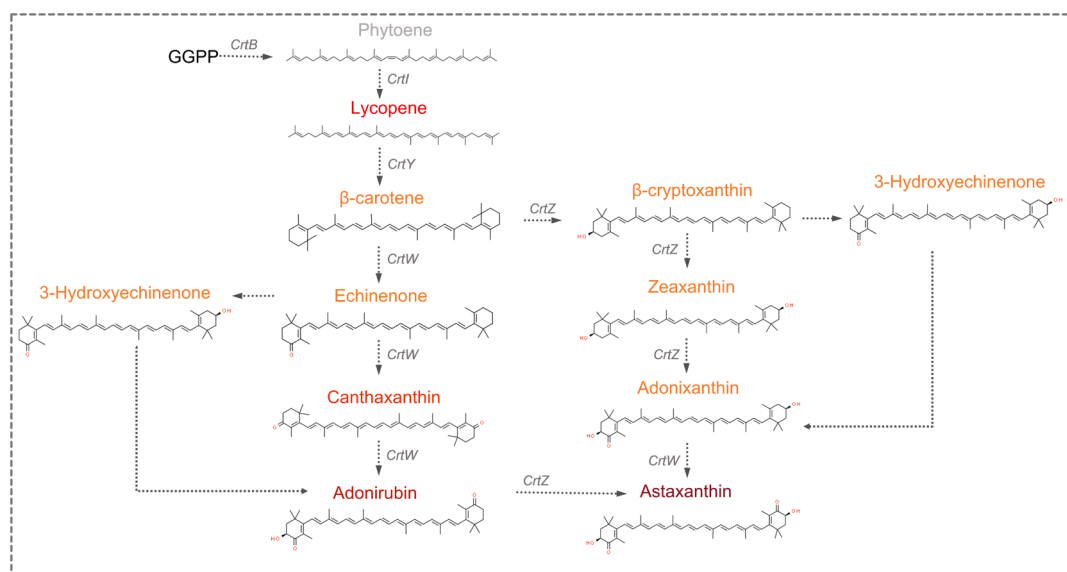


Fig. 2. Astaxanthin-biosynthesis pathway in *P. carotinifaciens* adapted from (Hayashi et al., 2021). Abbreviations: GGPP - geranylgeranyl pyrophosphate; Carotenogenic genes: *CrtB* (phytoene synthase); *CrtI* (phytoene desaturase); *CrtY* (lycopene cyclase); *CrtZ* (β -carotene hydroxylase) and *CrtW* (β -carotene ketolase).

1999). *P. carotinifaciens* is a motile, non-phototrophic, non-spore forming, rod-shaped and elongated colored-bacteria, characterized by the attractive round-orange colonies on nutrient agar (Tsubokura et al., 1999). Depending on the cultivation conditions, the size of a cell can vary (diameter from 0.3 to 1 μm and length from 1 to 5 μm) and the temperature for growing ranges from 25 to 30 $^{\circ}\text{C}$. It is notable that *P. carotinifaciens* bacteria are capable to use D-glucose, L-arabinose, maltose and D-xylose as a carbon source (Hayashi et al., 2021). Due to this nutritional versatility, *P. carotinifaciens* is being developed as an alternative green cell-factories to produce value-added compounds such as natural AXT for pharmaceutical, food, feed and cosmeceutical applications.

3. Astaxanthin biosynthesis in *P. carotinifaciens*

Several bacteria's have the capability to biosynthesis numerous types of natural carotenoids, however only the *P. carotinifaciens* bacterium is considered the most important source of AXT and two rare carotenoids (adonirubin and adonixanthin) with potential application in bio-refineries (Honda et al., 2021). *P. carotinifaciens* in particular demonstrates the ability to naturally biosynthesize AXT as the main carotenoid (~60 %), adonirubin (~25 %), canthaxanthin (~6%), adonixanthin (~3%), β -carotene (~2.5 %) and other carotenoids (~1%) (Hayashi et al., 2021). Note that the content of each carotenoid can vary according to the cultivation conditions. According to Ide et al. (2012) and

Table 1

Some examples of the AXT-producing *P. carotinifaciens* strains, cultivation conditions and AXT production yields.

| <i>P. carotinifaciens</i> strain | Cultivation conditions | | | AXT (mg/L) | References |
|----------------------------------|--|-------------------------------|------------|------------|-------------------------------|
| | Culture media (g/L) | Submerged culture | Time (day) | | |
| rNG5 | Glucose (5), Na_2HPO_4 (3.6), NaCl (8), NaH_2PO_4 (1.2), ammonium chloride (2.5) and yeast extract (2.5) | Stirred-tank 1.2 L bioreactor | 6 | 22 | 760 (Ide et al., 2012) |
| n.d | Sucrose (40), corn steep liquor (30), ammonium sulfate (0.5), potassium dihydrogen phosphate (2.25), disodium hydrogen phosphate dodecahydrate (5.7), calcium chloride dihydrate (0.1), magnesium sulfate heptahydrate (0.5) and iron sulfate heptahydrate (5) | Stirred-tank 5 L bioreactor | 2 | 28 | 596 (Katsumata et al., 2014) |
| E - 396 (FERM BP - 4283) | Sucrose (40), corn steep liquor (30), ammonium sulfate (0.5), potassium dihydrogen phosphate (2.25), disodium hydrogen phosphate dodecahydrate (5.7), calcium chloride dihydrate (0.1), magnesium sulfate heptahydrate (0.5), iron sulfate heptahydrate (5) and D - biotin (50) | Orbital shaker | 2 | 28 | 863 (Hirasawa et al., 2019) |
| E - 396 (FERM BP - 4283) | Corn steep liquor (30), potassium dihydrogen phosphate (1.5), disodium hydrogen phosphate dodecahydrate (3.8), calcium chloride dihydrate (5.0), magnesium sulfate heptahydrate (0.7) and iron sulfate heptahydrate (0.3) | Orbital shaker | 2 | 28 | 746 (Hirasawa et al., 2017) |
| LL1 (KP288668) | KH_2PO_4 (2), NaCl (25), $(\text{NH}_4)_2\text{SO}_4$ (2), K_2HPO_4 (8), yeast extract (6), tryptone (0.5), sodium citrate (2), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (2), $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$ (9), $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ (0.02) | Stirred-tank 1.5 L bioreactor | 1.5 | 30 | 10.2 (Khomlaem et al., 2021) |
| LL1 (KP288668) | Corn cob hydrolysate (20), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (9), KH_2PO_4 (0.5), $(\text{NH}_4)_2\text{SO}_4$ (1), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (1), $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ (0.02), NaCl (10), | Stirred-tank 1.5 L bioreactor | 4 | 30 | 11.95 (Khomlaem et al., 2023) |

Abbreviations: n.d: not described.

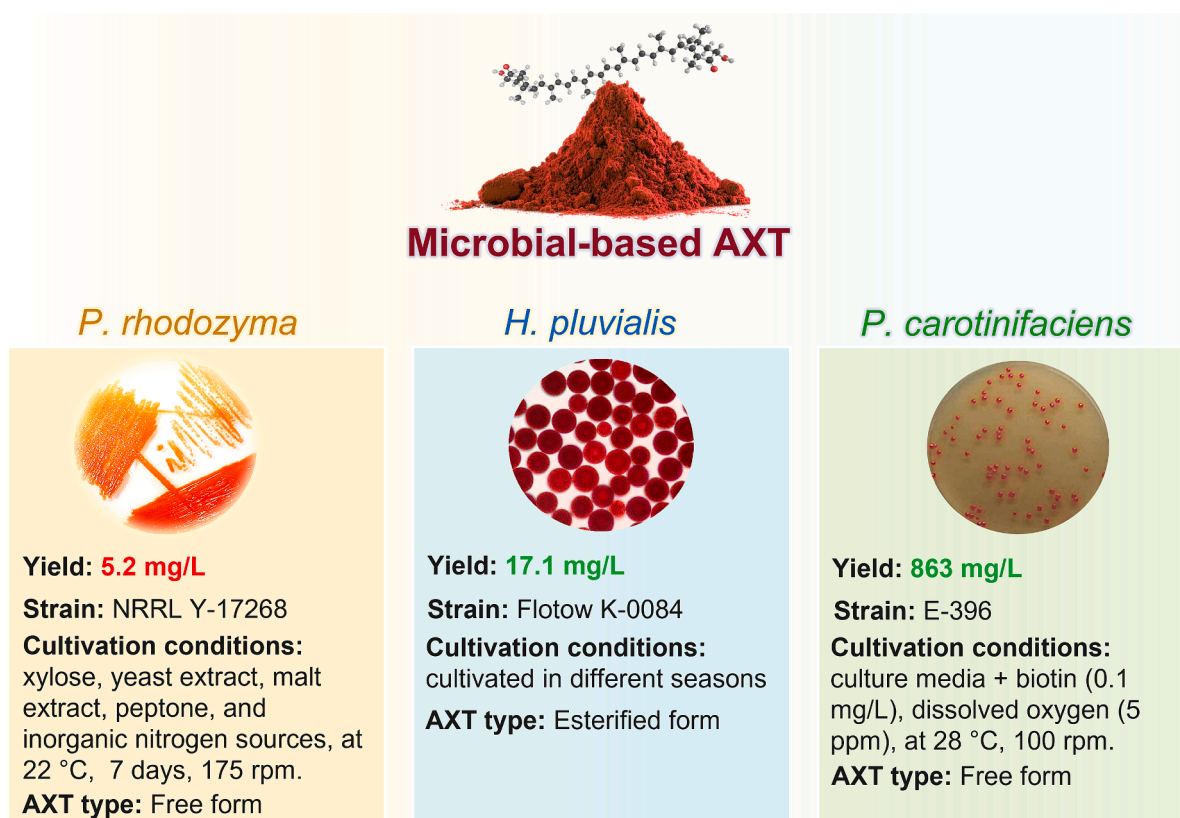


Fig. 3. Comparison of the main microbial producers of natural astaxanthin.

Hayashi et al. (2021) the AXT biosynthesis in *P. carotinifaciens* starts with the condensation of four isopentenyl pyrophosphate (IPP) – C₅ (the building block for the synthesis of C₄₀ carotenoids) into geranylgeranyl diphosphate (GGPP) – C₂₀. Two-molecules of GGPP – C₄₀ (C₂₀ + C₂₀) are then condensed to form colorless phytoene – C₄₀ by phytoene synthase (*CrtB*). The phytoene molecule is further converted into lycopene – C₄₀ by a series of unsaturation reaction through phytoene desaturase (*CrtI*). The red-carotene lycopene is subsequently converted into yellow/orange β-carotene through a cyclization reaction by lycopene cyclase (*CrtY*). The synthesis of several xanthophylls is performed introducing hydroxyl and keto groups into β-carotene using β-carotene hydroxylase (*CrtZ*) to obtain echinenone – C₄₀ and β-carotene ketolase (*CrtW*) to produce β-cryptoxanthin. The biosynthesis of AXT is performed after the synthesis of various xanthophylls such as canthaxanthin, adonirubin, zeaxanthin, among others as depicted in Fig. 2.

4. Production of astaxanthin by *P. carotinifaciens*

In the last years, the search for more biocompatible, simple, economic and efficient upstream and downstream procedures to produce natural carotenoids, including AXT have increased exponentially, in particular those that follow the biorefinery and bioeconomy precepts (Mussagy et al., 2021). Therefore, it is clear that the production of a specific type of AXT mainly depends by the microorganism used, the strain and cultivations conditions (*viz.*, carbon and nitrogen source, pH, agitation, cultivation time, among others) (Mussagy et al., 2019). The AXT produced by *P. carotinifaciens* differs from the other sources: (i) microalgae *H. pluvialis* produces (3S,3'S)-AXT mono- and diesters (more than 90 % of –OH group is conjugated with fatty acids) (Niizawa et al., 2021); (ii) yeast *P. rhodozyma* only biosynthesize (3R,3'R)-AXT conjugated or no with fatty acids (Mussagy et al., 2021a); and (iii) the AXT produced by *P. carotinifaciens* is characterized by the 3S,3'S-configuration in “free” form (Hirakida et al., 2022).

In fact, there are few research works performed on the production of AXT by *P. carotinifaciens* (Table 1), and among them, to understand the metabolic pathway some authors have used a simple synthetic cultivation media as a first strategy. In this context, Ide et al., (2012), enhanced the biosynthesis of AXT in *Paracoccus* sp. strain rNG5 using a culture media composed of (in g/L): glucose (5), Na₂HPO₄ (3.6), NaCl (8), NaH₂PO₄ (1.2), ammonium chloride (2.5) and yeast extract (2.5) in 1.20 L bioreactor at 22 °C, 600 rpm of agitation speed and air flow rate of 1.8 L/min. In this particular work, the authors revealed that recombinant strain reached 760 mg/L of AXT. Katsumata et al., (2014) carried-out AXT production studies using *P. carotinifaciens*, in 5 L stirred tank bioreactor in a culture medium composed of (in g/L): sucrose (40), corn steep liquor (30), ammonium sulfate (0.5), potassium dihydrogen phosphate (2.25), disodium hydrogen phosphate dodecahydrate (5.7), calcium chloride dihydrate (0.1), magnesium sulfate heptahydrate (0.5) and iron sulfate heptahydrate (5) at 100 rpm and 28 °C for 48 h, achieving 596 mg/L of AXT. Following the same cultivation conditions, Hirasawa et al. (2017) also evaluated the production of AXT using *P. carotinifaciens* strain E-396, obtaining 746 mg/L. The same authors (Hirasawa et al., 2019) have also reported a new patent with the improvement of AXT production (863 mg/L) by *P. carotinifaciens* strain E-396. Recently an innovative work for the concomitant production of AXT (11.95 mg/L) and polyhydroxyalkanoates (31.3 g/L) using *Bacillus megaterium* and *Paracoccus* sp. using corn cob hydrolysate as a carbon source was reported (Khomlaem et al., 2023).

Comparing the production of AXT by *P. carotinifaciens* with those obtained by Parajo et al., (1998) using yeast *P. rhodozyma* (5.2 mg/L) and Wang et al., (2013) using microalgae *H. pluvialis* (17.1 mg/L), these preliminary results indicated that *P. carotinifaciens* might be an interesting alternative for the industrial production of natural free from AXT (Fig. 3).

The comparison depicted in Fig. 3 highlights the significant difference between the production of AXT using *P. carotinifaciens*, *H. pluvialis*

Table 2
Examples of cell disruption and extraction procedures for the recovery of AXT from *P. carotinifaciens* strains.

| <i>P. carotinifaciens</i> strain | Type of Biomass | Cell disruption method | Solvents | SLR | Time (min) | Temperature (°C) | AXT recovery yields | References |
|----------------------------------|-----------------|--|---------------------------------------|---|------------|------------------|---------------------|--------------------------|
| n.d | Dried | Stirring | Hot ethyl alcohol (95–100 % in water) | n.d | n.d | n.d | 596 mg/g | (Katsumata et al., 2014) |
| N-81106 | Wet | Vortex + glass beads | Acetone-methanol (7:2, v/v) | 1 mL cells in culture media/ 0.4 mL solvent | n.d | n.d | 480 mg/L | (Ide et al., 2012) |
| n.d | Dried | Shaking + Z-isomerization-accelerating catalysts | Acetone + allyl isothiocyanate | n.d | 60 | 60 | 98 % | (Honda et al., 2020a) |
| ASB-5 | Dried | Supercritical CO ₂ + co-solvent | CO ₂ + Ethanol | 1 g/ 3 mL min ⁻¹ | 240 | 100 | 50 % | (Honda et al., 2022) |
| NITE SD 00017 | Dried | Stirring | DES (Lauric acid + caprylic acid) | 0.03 g/mL | 360 | 25 | 60 % | (Sapone et al., 2021) |
| n.d | Dried | Stirring + Switchable Hydrophilicity Solvent | N,N-dimethyl cyclohexylamine | 0.03 g/mL | 180 | 25 | 62.5 % | (Sapone et al., 2022) |

Abbreviations: SLR: biomass/solvent ratio; Recovery values in %: expressed as the amount of the extracted AXT; n.d; not described.

and *P. rhodozyma*. Furthermore, these results suggest that if the strategies that include: (i) the optimization of fermentation conditions to make the production of AXT by *P. carotinifaciens* more efficient and cost-effective; and (ii) the use of low-cost raw materials (i.e., agro-residues) and chemicals, are required for the industrial production of AXT using this microorganism. In this section, some aspects regarding the production of natural AXT by *P. carotinifaciens* and the comparison with other microbial sources were briefly discussed. However, despite the hyper-production of AXT using *P. carotinifaciens*, the biosynthesis of AXT is intracellular (the molecule remains inside of the cell) and for the recovery, appropriate procedures for the bacterial cell wall disruption are necessary for the successful release. Note that, the type and number of downstream units to recovery AXT are dependent on the final application, viz., for feed application for example, the whole biomass is used in the formulation. In the next section, we will describe some approaches regarding the downstream units used for the cell wall disruption and recovery of *P. carotinifaciens*-based AXT.

5. Recovery of astaxanthin from *P. carotinifaciens*

As cited before, the production of AXT by *P. carotinifaciens* is intracellular, and like some intracellular biomolecules such as lipids, proteins and other carotenoids, after the production stage, the cell disruption stage is required for the efficient recovery and processing of AXT. The cell structure of *P. carotinifaciens* is similar to those of Gram-negative bacteria's and therefore has all the properties typical of these microorganisms (Ruhhal and Kataria, 2021). The cell of a *P. carotinifaciens* comprises three different regions, which includes a double membrane with a thin peptidoglycan cell wall (approximately 12 and 14 nm) and a periplasmic space rich in AXT between the cell wall and cytoplasmic membrane (Nokhal and Mayer, 1979). The cell wall is composed of lipopolysaccharides, porins, lipids and lipoproteins content and revealed a low resistance to physical disruption (Khomlaem et al., 2021). In fact, in this work we call the attention of scientific community to this significant advantage of using *P. carotinifaciens* compared to the yeast *P. rhodozyma* (cell-wall thickness of approximately 0.46 µm composed of mannoproteins, chitin, glucans and cytoplasmic membrane) (Miao et al., 2021) and microalgae *H. pluvialis* (cells with a thickness of 1.8–2.2 µm composed of pectin, agar, alginate, hemicellulose, fucans, proteins and other compounds) (Kim et al., 2022). Taking into account that the

downstream procedures require adequate technologies, in particular the cell disruption unit which is responsible for the significant part of production costs to obtain pure AXT, the recovery of AXT from *P. carotinifaciens* is easier compared to other microbial sources, thus reducing the energy requirements and consumables (Mussagy et al., 2022b).

After the production stage, the colored biomass rich in AXT is traditionally separated from the culture media using centrifugation unit (clarification stage), which is technically feasible, but still not economically viable. In fact, the centrifugation unit is costly process and overall, it increases the cost of downstream procedures (Mussagy et al., 2022b). At this moment, there is no harvesting method, that is both economically viable and efficient, however some alternatives such as filtration procedures, appear as the most promising approach to reduce the *P. carotinifaciens* harvesting costs. Further, to facilitate the cell disruption, an additional pretreatment process that facilitates sequential extraction of the intracellular AXT from *P. carotinifaciens* biomass is required to obtain dry biomass. Following the pretreatment, the dried biomass is subjected to conventional physical or chemical cell-disrupting procedures using volatile organic compounds (VOCs) (Kim et al., 2022). As abovementioned, *P. carotinifaciens* cells are more fragile than *P. rhodozyma* and *H. pluvialis* and consequently the cell disruption is simpler than of these microorganisms. The recovery of AXT from *P. carotinifaciens* can be easily achieved using pure or mixtures of bio-based solvents or by the combination of low-cost and biocompatible VOCs with maceration or few cycles of freezing-thawing procedures. Note that the selection of VOCs is a prerequisite to obtain high recovery yields, the adequate choice of non-polar solvents is particularly interesting because of their high AXT (non-polar compound) solubilizing potential, as well as their high capability to dissolve the biomass (Mussagy et al., 2022a). Further studies regarding the extraction using different types of biomasses, viz., wet cells are required in order to reduce the unit of the integrated processes, reduce the cost and improve the knowledge of these procedures.

The influence of solvents on the recovery of AXT from *P. carotinifaciens* is not well-established, because there are few works in the literature related to the AXT extraction procedures from this microorganism (Table 2). Katsumata et al., (2014) evaluated the extraction capability of hot ethyl alcohol (95–100 % in water) for the recovery of AXT from dried *P. carotinifaciens* whole cell powder. In this particular

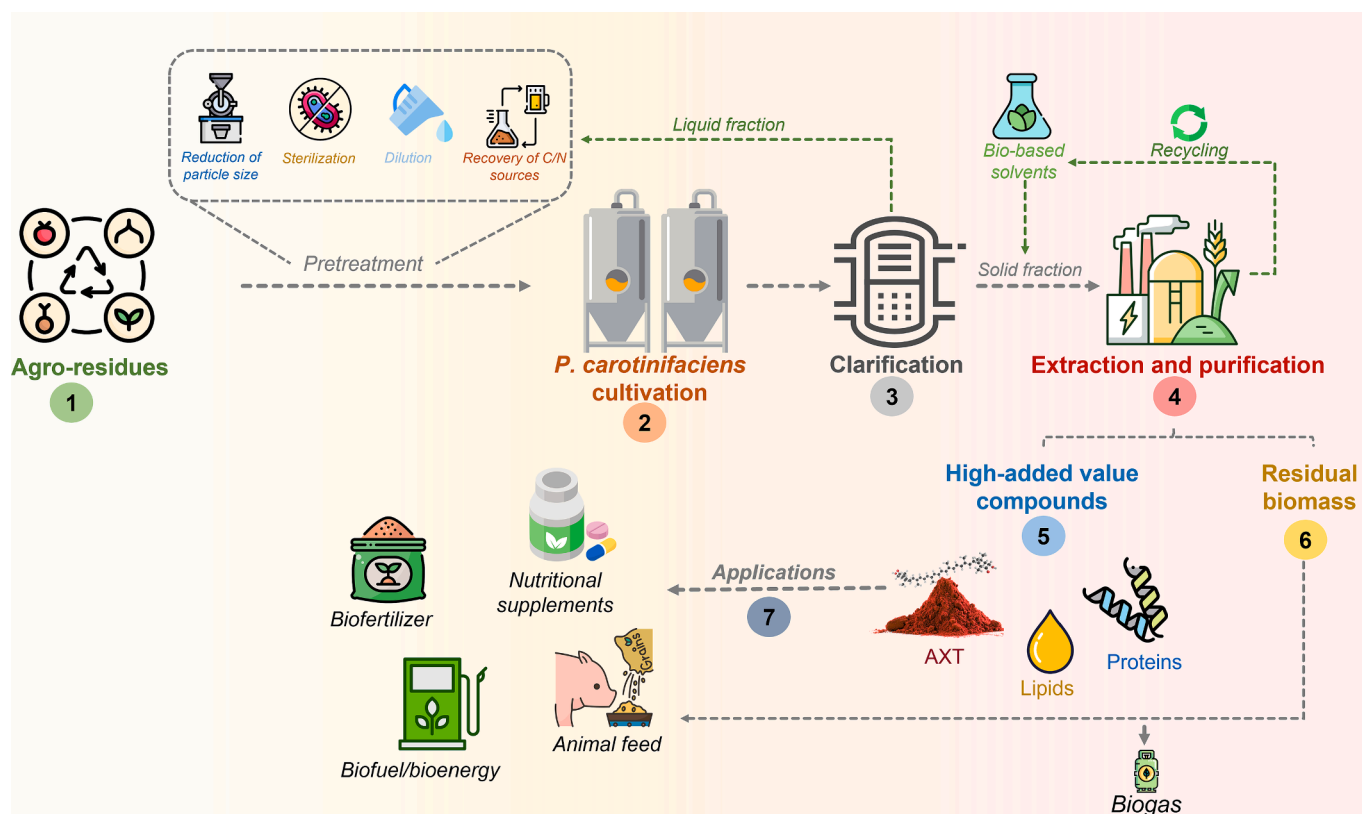


Fig. 4. Proposed *P. carotinifaciens*-based biorefinery processing plant for the production of astaxanthin and other high-added value biomolecules.

work, the authors also reported that the colored liquid mixture rich in AXT was cooled and filtered to remove the residual colorless biomass, and the colored filtrate was concentrated and crystallized, obtaining 596 mg/g of solid AXT. Similarly, Ide et al., (2012) evaluated the recovery of AXT using mixtures of VOCs [acetone-methanol (7:2, v/v)], reaching 480 mg/L of AXT. Honda et al., (2020a) described an efficient and ecofriendly procedure for the recovery of AXT from *P. carotinifaciens* using Z-isomerization-accelerating catalysts (allyl isothiocyanate at 60 °C) obtaining more than 98 % of AXT recovery. The same authors (Honda et al., 2022) evaluated the influence of supercritical CO₂ extraction + ethanol (co-solvent) on the recovery of AXT from *P. carotinifaciens*, achieving more than 50 % of recoveries. Recently, other classes of biocompatible solvents such as Switchable Hydrophilicity Solvent (SHS) and Deep Eutectic Solvents (DES), have been reported for the recovery of AXT from *P. carotinifaciens* in particular because of its low toxicity and eco-friendly nature (Sapone et al., 2022, 2021). Note that, the use of DES for the recovery of intracellular carotenoids is quite new, and so far, only one study has been performed on the recovery of AXT from *P. carotinifaciens* cells. In this work, AXT was obtained from dried biomass using DES composed of lauric acid and caprylic acid under stirring conditions in a thermostatic orbital shaker at 25 °C for 360 min, obtaining 60 % more AXT than those obtained with acetone (30 %) and ethanol (20 %) (Sapone et al., 2021).

In general, several combinations of VOCs and assisted-methods can be used for cell-disruption and recovery of AXT from *P. carotinifaciens* cells. The efficiency of each procedure will always dependent on the type of biomass, solid-liquid ratio, temperature, solvent time, concentration of solvent, and other operational conditions. Furthermore, we must always consider that before the selection of solvents and procedures, the techno-economic and life cycle assessment of the integrated process must be carefully evaluated. We particularly believe that the use of some neoteric solvents combined with innovative non-conventional assisted extraction procedures viz., supercritical fluids or ultrasound, will overcome some of the current ecological and processing problems,

making the process for the recovery of AXT from *P. carotinifaciens* more effective and ecofriendly.

6. Emerging applications of *P. carotinifaciens*

Most of the studies involving the production of AXT by *P. carotinifaciens* aim the development of technologies to improve the production for further application of the whole biomass in feed sector. However, the most promising and emerging applications of *P. carotinifaciens* are referred to the use of sustainable, accessible and simple technologies not only to improve the production of AXT, but also to obtain several biomolecules (including other carotenoids, enzymes and lipids), to be applied in several emerging sectors that range from biomaterials to the production of delivering therapeutic compounds. The next subsections describe some examples of the most interesting applications of *P. carotinifaciens*, as well as some perspectives to overcome its current limitations.

6.1. Biorefinery application of *P. carotinifaciens*

The composition of *P. carotinifaciens* biomass can vary significantly according to the cultivation conditions. The composition of dried commercial *P. carotinifaciens* have the following composition (% w/w): AXT (2.1), adonirubin (0.9), canthaxanthin (0.2), adonixanthin (0.1), β-carotene (0.08), asteroidenone (0.07), echinenone (0.05), 3-hydroxyechinenone (<0.01), other carotenoids (<0.01), crude protein (44.3), crude fat (7.5), crude fiber (<0.1), crude ash (7.0), nitrogen free extract (34.2) and moisture (3.5) (Panaferd, 2022). As observed, *P. carotinifaciens* have the ability to accumulate significant amounts of several biomolecules, including AXT, other carotenoids, proteins, lipids, and polysaccharides, which can also be exploited as “natural nutrients” for commercial applications. However, it is clear that the production of a single biomolecule (cf., AXT) in a microbial-based biorefinery is not sufficient for economic sustainability due to the high production costs

which can significantly affect the final price of the product (Mussagy et al., 2022b).

To date, most commercial *P. carotinifaciens* processes have focused only to obtain the whole biomass rich in AXT for direct application in feed formulations (Panaferd, 2022). However, an alternative approach for the valorization of *P. carotinifaciens*, is related to the implementation of *P. carotinifaciens*-based biorefineries following the circular economy percepts, in which we can obtain not only AXT as a single product, but a plethora of other high-added value compounds (carotenoids, lipids and proteins) with several applications in food, feed, pharmaceuticals and cosmeceuticals formulations. Here, in Fig. 4, a schematic representation of possible biorefinery plant for the strategic production of multiple bioproducts including AXT as the main component is proposed with a focus on the upstream and downstream procedures.

As depicted in Fig. 4, a biorefinery approach can be easily accomplished by using agro-industrial residues as source of carbon, nitrogen and other nutritional sources required for the cultivation of *P. carotinifaciens* from which AXT and other high-added value biomolecules can be recovered using a combination of bio-based solvents (that can be easily recycled) with assisted-extraction methods following the circular economy percepts. In the first stage of the integrated process, for the efficient valorization of agro-industrial residues, a pre-treatment step is required to recovery hydrosoluble nutrients used in the cultivation stage, where the optimization of the cultivation parameters must be performed for the improvement of AXT production. The second step includes the clarification stage for the separation of the colored biomass-rich in AXT and other biomolecules from the culture media (that can be recycled for the new cultivation stage).

The colored *P. carotinifaciens* biomass is usually subjected to a drying pretreatment and then used in the extraction procedures. At this stage, due the characteristics of Gram-negative cell walls, the use of low-cost and biocompatible solvents (pure or mixed) such as ethyl acetate, isopropanol, ethanol, ethyl lactate, among others combined with non-conventional assisted procedures can be efficient alternatives for the sustainable recovery of intracellular biomolecules, minimizing the cost of the process. After the solid liquid extraction, the extract rich in AXT, lipids and proteins, can be subjected to a precipitation unit to separate proteins or saponification to isolate lipids for the application separately in the formulation of nutritional supplements, feed additives, among others. Note that the resulting colorless biomass can be used as a valuable raw material for the production of biogas or used directly in the formulation of feed additives. Furthermore, the solvents used in the extraction and purification stages, can be easily recycled by addition of an evaporation unit, and reused in the following extraction or purification procedures closing the circular economy approach.

For instance, the magnitude of the potential benefits of biorefinery of *P. carotinifaciens* to obtain AXT and other bioproducts will always be dependent of the environmental sustainability and techno-economic assessment of the process. Note that, the use of advanced sustainability assessment tools, including life cycle assessment (LCA), techno-economic analysis (TEA), energy, emergy and exergy evaluation, are required to establish the overall sustainability of the bioprocess in order to propose new solutions to mitigate the economic and environmental hot spots (Aghbashlo et al., 2022).

6.2. Markets scenario and applications of *P. carotinifaciens*

This section provides a detailed case study of an apparently successful application of *P. carotinifaciens* available in the market. The commercial *P. carotinifaciens* (dried biomass) is sold under the name Panaferd®-AX and Panaferd®-P for application in aquaculture and poultry, respectively, as feed additives used for salmon, trout's and eggs coloration (Panaferd, 2022). Panaferd®-AX or Panaferd®-P are considered the most innovative projects implemented by the ENEOS company (Japan) in accordance with their market demands. These commercial products are mainly composed of inactive dried,

nontoxicogenic and nonpathogenic *P. carotinifaciens* biomass rich in AXT and other compounds (lipids, proteins and other carotenoids) directly used as additive in the diet to improve the coloration and health of organisms. According to the EFSA, Panaferd®-AX revealed to be bioavailable and effective in coloring of salmon and rainbow trout, however the deposition in flesh was less efficient compared to the synthetic-AXT. Furthermore, organoleptic properties of fishes treated by Panaferd®-AX were not different from those treated with synthetic-AXT (EFSA, 2007). Honda et al., (2020b) evaluated the potential of Panaferd®-P to improve egg yolk pigmentation of laying hens. In this work, the authors revealed that feeding hens with Panaferd®-P increased the concentrations of AXT, adonirubin and adonixanthin and also improved the egg yolk pigmentation, proving that the supplementation with this product can increase the nutritional value of hens' egg yolk. Recently, Nuntapong et al. (2022), evaluated the effects of Panaferd®-AX on growth, survival, pigmentation, disease resistance and immune response in white shrimps, revealing that the supplementation of shrimps with Panaferd®-AX increases the disease resistance against bacteria's and virus and also improved the pigmentation and antioxidant capacity of shrimps.

As observed the application of whole biomass of *Paracoccus* rich in AXT as feed additive is well-established (Panaferd, 2022), however considering the biological activity of AXT and its possible applications as anticancer, antioxidant, antidiabetic agent (Mussagy et al., 2021b), the *P. carotinifaciens*-based AXT can also be used in food formulations for human consumption. At the moment, the exclusive microbial source of natural AXT that received approval from the FDA and EFSA for use as human nutritional supplement is *H. pluvialis* (Jannel et al., 2020). However, AXT obtained from *H. pluvialis* represent approximately less than 1 % of the total world production with market price estimated at USD 7000 per kg due to the high processing costs (Novoveská et al., 2019). With the increase of the market demand for natural AXT, the development of new alternative sources of natural AXT are crucial not only to improve the production titers but also to supply the market with low-cost natural AXT. *P. carotinifaciens*-based AXT has not been implemented in the market for food or diverse therapeutic applications, mainly due to the not-well established processes and lack of scientific information's regarding the safety of *P. carotinifaciens*-based AXT products in human health. It is clear that these studies can provide critical information for the regulatory approval of this product, however the safety and efficacy of *P. carotinifaciens*-based AXT must be assessed and approved by the regulatory agencies (FDA or EFSA) before the implementation in the market. Some requirements including, characterization of the microbial strain (genome sequence data), tolerance studies of target species, toxicological profile of the active AXT are required to demonstrate the safety of the new microbial-based AXT for human health and consequently establish the new product in the market. Furthermore, envisioning the use of *P. carotinifaciens*-based AXT in therapeutic and biomedical applications, and also taking into account the low bioavailability, the sensitivity to environmental conditions and the lack of an adequate drug delivery system, an extensive effort must be performed to increase the bioavailability, stability, and solubility of this microbial-based powerful antioxidant. Among the various methods used for AXT formulation, nanoencapsulation emerges as an efficient tool to improve the physicochemical properties and therapeutic effects for subsequent application in cardiovascular and liver diseases, cancer treatment, protection of the skin and eyes against UV radiation, among others.

7. Concluding remarks and perspectives

The increase of consumers preference for natural products (i.e., AXT) has grown exponentially over the last few years (Ren et al., 2021). The search for new microbial sources, viz., *P. carotinifaciens* and the development of advanced upstream and downstream technologies are necessary not only to improve the production yields but also to lead to

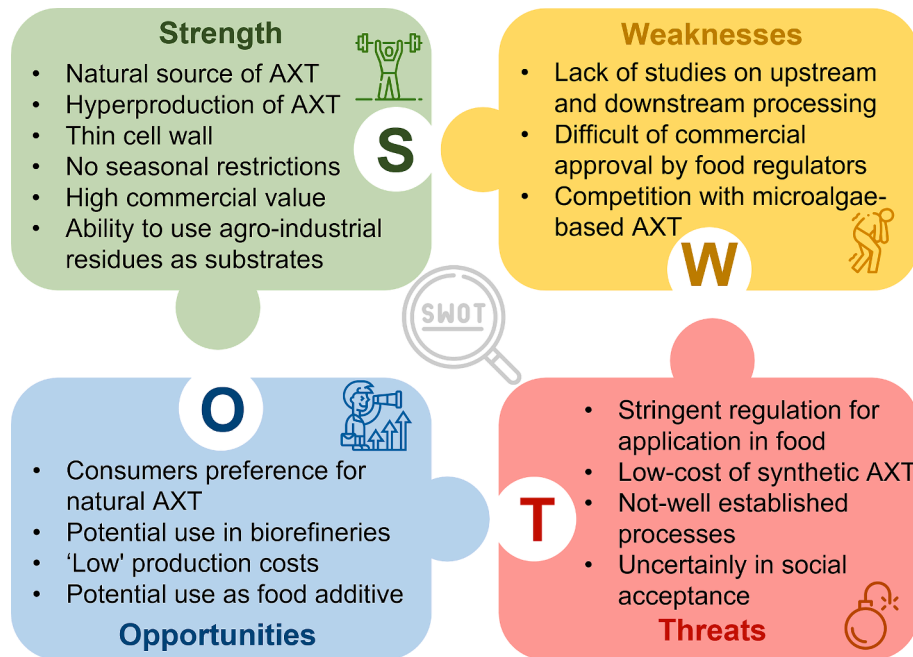


Fig. 5. SWOT analysis of production of astaxanthin from *P. carotinifaciens*.

lower product development costs and meet the huge demand for natural carotenoids in the food, cosmeceutical, pharmaceuticals and feed industries. To understand the scenario of *P. carotinifaciens*-based AXT, in Fig. 5 a SWOT analysis is performed to understand the strengths, weaknesses, opportunities and threats in order to obtain a panoramic viewpoint of the commercial viability of AXT produced by *P. carotinifaciens*.

As aforementioned, the main strengths of *P. carotinifaciens* are stated by the hyperproduction of AXT, its low media requirements due to the ability to use agro-industrial residues as substrates for the production of natural AXT at low-cost, no seasonal limitations, and great commercial value. The production and recovery of other compounds with high-added value such as lipids, enzymes, among others, add value to the entire process and consequently reduce manufacturing costs. The insufficiency of studies on upstream and downstream processes, difficult of commercial approval by food regulators and competition with microalgae-based AXT represent the main weaknesses of the commercial application of *P. carotinifaciens*-based AXT in food industries. Besides that, there is also a large market opportunity in the potential use of *P. carotinifaciens* in biorefineries. Consumers preference towards natural AXT and environmental safety has improved the interest in this technology which can open new market opportunities for application in food or pharmaceutical industries. As also described in Fig. 5 natural *P. carotinifaciens* -based AXT for use in the food industries are challenging to the market, mainly due to the stringent regulations of FDA and EFSA, low-cost of synthetic AXT, not-well established processes and uncertainty in consumers acceptance.

In general, considering the high AXT production by *P. carotinifaciens* compared to other microbial sources, the application of bacterial-based AXT seems to be a promising alternative source for further application in food industry envisioning the human consumption. However, some regulatory challenges still need to be overcome, and for that, the interdisciplinary scientific developments involving, chemical engineers, molecular biologists, bioprocess technologists, specialists in microbiology and toxicology are required to obtain further scientific information to prove the safety and applicability of *P. carotinifaciens* -based AXT to food regulators. Furthermore, the life cycle and techno-economic assessments of the proposed technologies must be assessed before the implementation in the market in order to construct a biocompatible, safe

and economic viable AXT source.

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Cassamo U. Mussagy: Conceptualization, Visualization, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. **Laurent Dufossé:** Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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