



Importance of Downstream Processing of Natural Astaxanthin for Pharmaceutical Application

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Astaxanthin (ASX) is a xanthophyll pigment considered as a nutraceutical with high antioxidant activity. Several clinical trials have shown the multiple health benefits of this molecule; therefore, it has various pharmaceutical industry applications. Commercial astaxanthin can be produced by chemical synthesis or through biosynthesis within different microorganisms. The molecule produced by the microorganisms is highly preferred due to its zero toxicity and superior therapeutic properties. However, the biotechnological production of the xanthophyll is not competitive against the chemical synthesis, since the downstream process may represent 70–80% of the process production cost. These operations denote then an opportunity to optimize the process and make this alternative more competitive. Since ASX is produced intracellularly by the microorganisms, high investment and high operational costs, like centrifugation and bead milling or high-pressure homogenization, are mainly used. In cell recovery, flocculation and flotation may represent low energy demanding techniques, whereas, after cell disruption, an efficient extraction technique is necessary to extract the highest percentage of ASX produced by the cell. Solvent extraction is the traditional method, but large-scale ASX production has adopted supercritical CO₂ (SC-CO₂), an efficient and environmentally friendly technology. On the other hand, assisted technologies are extensively reported since the cell disruption, and ASX extraction can be carried out in a single step. Because a high-purity product is required in pharmaceuticals and nutraceutical applications, the use of chromatography is necessary for the downstream process. Traditionally liquid-solid chromatography techniques are applied; however, the recent emergence of liquid-liquid chromatography like high-speed countercurrent chromatography (HSCCC) coupled with liquid-solid chromatography allows high productivity and purity up to 99% of ASX. Additionally, the use of SC-CO₂, coupled with two-dimensional chromatography, is very promising. Finally, the purified ASX needs to be formulated to ensure its stability and bioavailability; thus, encapsulation is widely employed. In this review, we focus on the processes of cell recovery, cell disruption, drying, extraction, purification, and formulation of ASX mainly produced in *Haematococcus pluvialis*, *Phaffia rhodozyma*, and *Paracoccus carotinifaciens*. We discuss the current technologies that are being developed to make

downstream operations more efficient and competitive in the biotechnological production process of this carotenoid.

Keywords: Astaxanthin extraction, astaxanthin formulation, astaxanthin purification, cell disruption, cell recovery, *Paracoccus carotinifaciens*, *Phaffia rhodozyma*, *Haematococcus pluvialis*

INTRODUCTION

Astaxanthin (ASX) (3, 3'-dihydroxy- β,β -carotene-4, 4'-dione) is a natural red pigment that serves as a precursor of vitamin A (Singgih and Julianti, 2015). Due to its potent antioxidant activity (superior to α , β -carotene, lutein, lycopene, canthaxanthin, and vitamin E), astaxanthin has gained considerable popularity as a human dietary supplement (Cuellar-Bermudez et al., 2015). Various investigations have provided substantial evidence of the use of this compound for various health conditions like prevention and treatment of atherosclerosis, cancer chemoprevention, maintenance and treatment of eye and vision disorders, anti-inflammatory properties, dermatological conditions, among others (Table 1) (Williamson et al., 2020). The multiple health benefits that have been reported of ASX make this molecule a real alternative in the pharmaceutical industry for the prevention and treatment of several diseases.

Currently, industrial production of ASX is carried out mainly by chemical synthesis; however, the biosynthesis of this molecule, by the cultivation of microalgae, yeast, and bacteria, is gaining industrial importance. This shift occurs mainly due to safety concerns for human consumption and higher biological activities of the biosynthesized compound than the chemically prepared molecule (Diao et al., 2020). Unfortunately, the biotechnological process for ASX production is not economically competitive against chemical synthesis, so the improvement of ASX production, mainly the downstream processes, has become a significant scientific challenge, regardless of the microorganism used to produce this molecule. The downstream processes represent up to 80% of ASX's production costs, so any improvement in these stages may have a considerable impact on the whole feasibility of the process.

Since ASX is produced intracellularly, cell recovery and cell disruption processes are the initial stages in the downstream process, being determinant in the process's total yield. Most companies employ sedimentation and centrifugation to concentrate the biomass in ASX's biotechnological production (Li et al., 2020b). However, new and more economical alternatives like flocculation and flotation have been proposed in the dewatering process of the biomass (Barros et al., 2015). Cell disruption is a necessary step in the obtention of the metabolite and can be carried out by classical physical, chemical, or enzymatic methods, with the use of ionic liquids in recent years, since they are considered green solvents and proved to be useful for cell disruption and ASX extraction (Choi et al., 2019). The use of a specific method may depend on the producing microorganism since bacteria, yeast, and microalgae differ in their cell wall and cellular membrane composition (Gomes et al., 2020).

After cell disruption and to avoid ASX degradation before the extraction process, spray drying or freeze-drying are generally used. The operational parameters must be carefully selected when using these technologies since ASX is a molecule with low stability and can be degraded during the drying process and storage of the powder (Ahmed et al., 2015). Similarly, the drying process may be used when formulating commercial ASX products, so the product stability must also be ensured (Feng et al., 2018; Zhao et al., 2019).

The ASX extraction from dried, cracked cells is carried out mainly using organic solvents due to the hydrophobic nature of ASX. However, there are other innovative approaches to improve the extraction process, such as combining cell disruption and ASX extraction into a single step and the use of compressed fluids technologies like supercritical fluid extraction (Molino et al., 2020) and pressurized liquid extraction (Gallego et al., 2019).

TABLE 1 | Recent reports on astaxanthin health benefits.

Model	Health benefit	References
Human gastric adenocarcinoma cell lines AGS, KATO-III, MKN-45, and SNU-1	Suppression of the cancer cell lines viability and inhibition of proliferation in KATO-III and SNU-1	Kim et al. (2016)
Mice strains C57BL/6J and ApoE ^{-/-}	Prevention and treatment of atherosclerosis	Zou et al. (2017)
Human retinal pigment epithelial cell line	Treatment of macular degeneration	Musa et al. (2019)
Macrophages cell lines	Anti-inflammatory and antioxidant activity	Farruggia et al. (2018)
RAW264.7, BMDM of WT, and <i>Nrf2</i> -deficient mice		
Incisional wound model in Wistar rats	Wound healing and tissue regeneration	Veeruraj et al. (2019)
Type 2 diabetes individuals	Improvement in glucose metabolism and reduction of systolic blood pressure and hyperglycemia	Mashhadi et al. (2018)
Mice strain		
C57BL/6	Improvement in lipid metabolism and gut microbiota	Wang et al. (2019)
Sea bass fish (<i>Dicentrarchus labrax</i>)	Immunostimulant and antioxidant	Saleh et al. (2018)
Epithelial cells	Prevention of inflammatory process and carcinogenesis-related signaling pathways	Kim et al. (2019)
AGS	produced by <i>Helicobacter pylori</i>	

Other assisted technologies like ultrasound-assisted, microwave-assisted, enzyme-assisted, and pulsed electric field-assisted are being extensively tested (Saini and Keum, 2018).

After extraction, the purification step is essential when preparing nutraceutical or pharmaceutical products since a high-purity product is required (99% or more). The liquid-solid chromatography is extensively used in ASX purification (Yang et al., 2017; Casella et al., 2020), whereas liquid-liquid chromatography may simplify and accelerate the process (Du et al., 2016; Fábryová et al., 2020). The parameters that influence the ASX purification in chromatography can be optimized to improve the process even though processing time is always the most difficult to reduce. On the other hand, the use of new techniques like comprehensive two-dimensional liquid chromatographies may considerably reduce sample processing time (Gallego et al., 2019).

The formulation is the final step in the downstream process of ASX. This molecule is relatively unstable due to its intrinsic reactivity; therefore, the formulation must ensure the purity, stability, and delivery of the compound in the best possible form. Encapsulation is the preferred method in ASX formulation, where different conventional techniques are extensively reported (Santos-Sánchez et al., 2020); however, modernization can be seen in the use of SC-CO₂ to produce capsules of the carotenoid (Liu et al., 2019a).

Since ASX has shown great potential for the pharmaceutical industry, the need for a competitive biotechnological process is greatly desired. The present review focuses on the downstream process for the obtention of ASX, where we analyze the current technologies and recent advances that represent cheaper or more efficient alternatives. Because the microbial production of ASX can be carried out in different microorganisms, the cell disruption and extraction methods can vary from one organism to another. Also, the characteristics of the final product differ substantially, and the purification step may require pretreatment. Other reviews discussed the ASX production process in the microalga *Haematococcus pluvialis*, but we emphasize studies developed in microorganisms of industrial interest and accepted by the Food and Drug Administration (FDA) for ASX production. Specifically, we discuss *H. pluvialis*, *Phaffia rhodozyma*, and *Paracoccus carotinifaciens* in the biosynthesis and mainly the new insights in the downstream process for the production of ASX molecule.

THE ASX MOLECULE

Carotenoids are terpenoid pigments of 40 carbon atoms, classified into carotenes and xanthophylls. Carotenes, like β -carotene and torulene, only have carbon and hydrogen in their chemical structure, while xanthophylls, like ASX and canthaxanthin, also contain oxygen (Mata-Gómez et al., 2014).

ASX has the molecular formula C₄₀H₅₂O₄, and its molar mass is 596.84 g/mol. It is a ketocarotenoid belonging to the chemical group of terpene compounds insoluble in water but soluble in most organic solvents. Its systematic IUPAC name is (6S)-6-hydroxy-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-[(4S)-4-hydroxy-2,6,6-

trimethyl-3-oxocyclohexen-1-yl]-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-2,4,4-trimethylcyclohex-2-en-1-one. It is known by astaxanthin, E-astaxanthin, (S,S)-astaxanthin, astaxanthin, and all-trans-astaxanthin (PubChem, 2020). This carotenoid contains eight successive condensed isoprene molecules and 11 conjugated double bonds (polyene system). The two ends of the ASX molecule have a β -ionone ring with hydroxy (OH) and keto (C=O) groups (**Figure 1**); these characteristics of the molecule can explain its higher antioxidant activity and its ability to be esterified (Ambati et al., 2014).

The double bonds present in ASX are critical in the antioxidant capacity of this carotenoid. They react and donate electrons to free radicals, neutralizing and converting them into stable molecules (Guerin et al., 2003). When one hydroxyl group reacts with a fatty acid, a monoester is formed, whereas when both hydroxyl groups react with fatty acids, a diester is formed (Ambati et al., 2014). Due to its chemical structure, ASX is sensitive to light, heat, and oxygen and therefore can quickly occur during extraction, isomerization, or degradation reactions (Fang et al., 2019). Like the esterification by one or two fatty acids, other reactions produce more stable structures than ASX free form (Hussein et al., 2006). ASX may also form a chemical complex with proteins (carotenoproteins) or lipoproteins (carotenolipoproteins) (Higuera-Ciapara et al., 2006).

The molecule of ASX displays geometric isomers *cis* or *trans* because each double bond from the polyene chain may exist in the two configurations (Higuera-Ciapara et al., 2006; Visioli and Artaria, 2017). We can find the isomers *E-trans* or *Z-cis* in ASX, the most common being all-*E-trans* ASX, 13-*Z-cis* ASX, and 9-*Z-cis* ASX (Honda et al., 2020b). Although *cis*-isomers are thermodynamically less stable than the *trans*-isomers, the *cis*-configurations have shown higher antioxidant power and bioactivity than that of the *trans*-configuration (Liu et al., 2018b; Yang et al., 2019).

All-*E-trans* ASX might exist as three stereoisomers since it contains chiral carbon atoms with R or S stereochemistry in their 3 and 3' positions of the β -ionone rings. The three possible optical isomers of astaxanthin are the enantiomers 3R, 3'R and 3S, 3'S, and the meso form 3R, 3'S (**Figure 1**). Different antioxidant activities have been observed in the enantiomers of ASX, and it has been reported that 3S, 3'S-*trans* form exhibits higher bioactivity than the other stereoisomers (Liu et al., 2016; Sun et al., 2016).

NATURAL ASX AND SYNTHETIC ASX, PRICE COMPETITIVENESS

According to Zhang et al. (2020a), ASX consumption has increased dramatically in the last five years. Only natural astaxanthin (N-ASX) can be used as a human nutraceutical supplement due to several safety studies, extensive clinical trials, and regulations. This carotenoid is obtained from natural sources such as bacteria, protists, fungi, algae, plants, and crustacean by-products (Fang et al., 2019; Tran et al., 2019; Zhang et al., 2020a). Synthetic astaxanthin (S-ASX), on the other

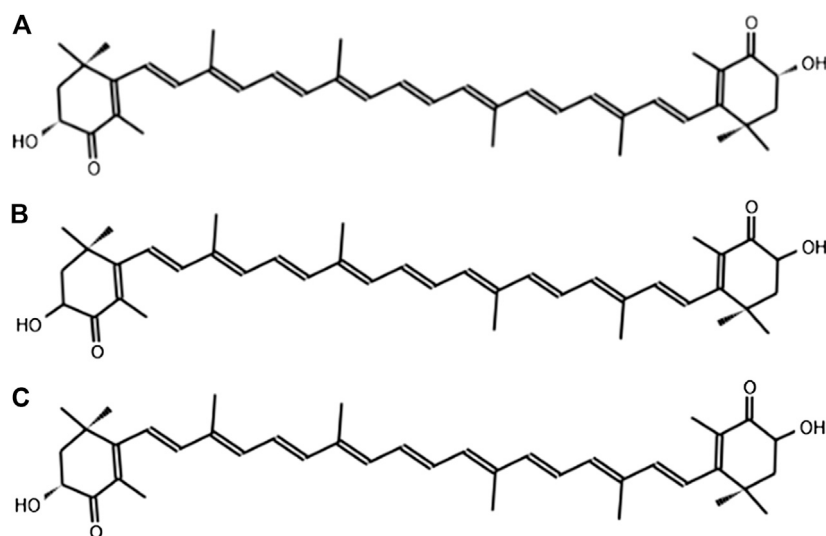


FIGURE 1 | Stereoisomers of the chemical structure of astaxanthin: **(A)** 3R,3'R; **(B)** 3S,3'S; **(C)** 3R, 3'S.

hand, has not been proven safe for human consumption, and its use is only limited as a feed ingredient to pigment the flesh of fish in aquaculture (Capelli et al., 2013). S-ASX is synthesized in a multistep process from petrochemicals. Further, N-ASX has shown no toxicity and has demonstrated superior therapeutic properties than S-ASX (Shah et al., 2016).

Commercial ASX is produced by the chemical method, S-ASX, or biosynthesis, N-ASX, in the alga *Haematococcus pluvialis*, the yeast *Phaffia rhodozyma*, and the bacteria *Paracoccus carotinifaciens* (Ambati et al., 2014; Katsumata et al., 2014). However, other microorganisms are promising for the biotechnological production of N-ASX (Sun et al., 2019; Tran et al., 2019). N-ASX has become a compound of great interest in the pharmaceutical industry, and many companies are producing this carotenoid (Table 2).

Despite N-ASX's advantages, about 99% of the ASX on the market is produced synthetically by big companies such as BASF and Hoffman-La Roche, while less than 1% is derived from natural sources (Molino et al., 2018b). In recent years, the market for N-ASX has been growing substantially. The market value of N-ASX depends on the purity of the product and varies from USD 2,500–7,000/kg to about USD 15,000/kg in the case of N-ASX for human applications such as cosmetics, food, and dietary supplements. S-ASX has a market value of about USD 1,000/kg (Koller et al., 2014; Shah et al., 2016; Molino et al., 2018b; Zhang et al., 2020a). Rodríguez-Sáiz et al. (2010) reported a price of USD 2,500/kg for pure N-ASX and suggested using improved strains of *P. rhodozyma*, low-cost raw materials, and the improvement of fermentation parameters may provide a cost-effective alternative method in preparing N-ASX. Panis and Carreon (2016) used a theoretical model to perform a technical and economic study for N-ASX production from *H. pluvialis* in two European cities: Livadeia (Greece) and Amsterdam (Netherlands). In that report, N-ASX production cost (not considering the downstream process) was calculated at

€1,536/kg and 6,403/kg for Livadeia and Amsterdam, respectively, while the production cost for S-ASX was €880/kg. The authors concluded that N-ASX is yet unable to compete with S-ASX under the analyzed conditions.

New strategies must be sought to make N-ASX production more competitive, and the downstream processes must be considered due to their high impact on the overall cost of the process as previously described. Zgheib et al. (2018) reported a theoretical economic study for extracting N-ASX from *H. pluvialis*. In this study, the authors assumed that the biomass suspension was supplied from the microalgae producing platform; therefore, they focused on the costs generated in the downstream process. Economic viability was achieved with an N-ASX price superior to USD 1,500/kg and biofertilizer's commercialization obtained from the residual *H. pluvialis* biomass, estimated at USD 40/kg, which added value to the process. Li et al. (2011) operated an N-ASX production pilot plant from which they scaled up to a hypothetical plant with a production capacity of about 900 kg N-ASX per year. According to their economic analysis, including the cultivation of *H. pluvialis* and the downstream process, the production cost of the carotenoid was USD 555/kg or USD 718/kg, considering a 10 years depreciation. In contrast, the production cost of S-ASX was estimated to be about USD 1,000/kg. The authors attributed N-ASX production's low-cost to a low-cost photobioreactor employed, two-step cultivation of *H. pluvialis*, cheap labor, land, and utilities in China. Furthermore, this economic analysis was carried out based on biomass with 2.5% N-ASX and the authors mentioned that this concentration might be higher in the microalgae, so production costs would also decrease. Dursun et al. (2020) simulated two processes to obtain powdered N-ASX from wheat bran and oil mixture from olive pomace. The N-ASX was produced in solid-state fermentation by the cultivation of *P. rhodozyma* in the two agro-industrial wastes. The authors integrated the upstream and downstream processes

in their economic analysis and calculated a production cost of USD 13,870/kg for the powder formulation (PF), whereas for the oil mixture formulation (OF), it was estimated at USD 7,809/kg. The two processes proved to be feasible, and the payback times of PF and OF processes were 4.92 and 7.19 years, respectively.

Replacing S-ASX for animal feed application with N-ASX is very attractive due to the superior properties of N-ASX. The main holdback is clearly the price of N-ASX, which may reach USD 5,000/kg whereas the S-ASX price is still only about USD 2,000/kg (Li et al., 2020b). On the other hand, although N-ASX generally has a higher production cost than S-ASX, its value may be overpriced since its market niche is directed to treating diseases.

RELEVANT BIOTECHNOLOGICAL SOURCES FOR N-ASX PRODUCTION

Since N-ASX can be biotechnologically produced principally by *H. pluvialis*, *P. rhodozyma*, and *P. carotinifaciens*, it is of great interest to examine the characteristics of these microorganisms to understand the downstream processes carried out in each different cell type. Alternatively, many genetically modified organisms (GMO) have been generated and may represent a more efficient option to produce this red pigment.

Haematococcus pluvialis

H. pluvialis is the only authorized N-ASX-producing microorganism for human consumption. N-ASX obtained from this microalga has been approved in Europe, Japan, and the United States. The European Food Safety Authority (EFSA) has advised an acceptable daily intake of 0.034 mg/kg for humans, whereas the United State Food and Drug Administration (FDA) considers N-ASX from *H. pluvialis* as “GRAS” (generally recognized as safe) (Davinelli et al., 2018). *H. pluvialis* (class Chlorophyceae; order Volvocales; family Haematococcaceae) is a unicellular freshwater green microalga worldwide (Eom et al., 2006). It is a photosynthetic eukaryote cell with spherical, ellipsoidal, or pear-shape with two flagella when it grows under favorable cultural conditions. This type of morphology is called macrozooids (zoospore); the cells are green and between 8 and 20 µm long (Hagen et al., 2002). The macrozooids are fast-growing vegetative cells and may divide into 2–32 daughter cells by mitosis (Shah et al., 2016). When environmental stress occurs (i.e., nutrient deprivation, high salinity, nonoptimal temperature, or high light irradiance), the cells interrupt cell division, lose their flagella (they transform from motile to nonmotile cells), and increase their cell size. This morphology is known as hematocysts (aplanospores), and during that stage, the microalgae accumulate large amounts of astaxanthin and lipids, acquiring a characteristic bright red color (Hagen et al., 2002). Aplanospore cell walls are composed of an outer primary wall, a trilaminar sheath, a secondary wall, and a tertiary wall. The trilaminar sheath contains sporopollenin-like material (algaenan), while the secondary and tertiary walls contain mannose and cellulose. These structures give the cells high resistance against extreme environmental conditions and the ability to withstand

mechanical and chemical attacks. Thus, the *H. pluvialis* structure makes it difficult to extract the N-ASX (Hagen et al., 2002; Safi et al., 2014; Liu et al., 2017). This microalga is considered the best source of N-ASX since it can accumulate 3.8–5.0% dry weight of this carotenoid, depending on the culture conditions, producing predominantly esterified N-ASX (95%) in the 3S, 3'S form (Chekanov et al., 2014; Khoo et al., 2019a).

Phaffia rhodozyma

P. rhodozyma was considered the only native N-ASX producing yeast until *Rhodospodium toruloides* was recently reported (Tran et al., 2019). *P. rhodozyma* (class Tremellomycetes; order Cystofilobasidiales; family Cystofilobasidiaceae), also known as the teleomorphic state *Xanthophyllomyces dendrorhous*, is considered the second-best source of biotechnological production of N-ASX and produce mainly free and nonesterified 3R, 3'R N-ASX (Kucsera et al., 2000). This yeast is considered as GRAS microorganism and approved as a color stabilizer for fish feed supplementation by the FDA (Barredo et al., 2017). *P. rhodozyma* was initially isolated from slime fluxes from broad-leaved trees in Japan by Herman J. Phaff. Ten strains of this yeast were isolated in the late 60's from *Alnus*, *Betula*, *Cornus*, *Fagus*, and *Ulmus* tree types. Later, it was also found in Russia, Chile, Finland, and the United States. The vegetative cells of *P. rhodozyma* are ellipsoidal, reproduce by budding, and occur singly, in pairs and sometimes in short chains. Sexual activity has been observed in this yeast, involving mating and sporulation between two yeast cells. The conjugation between the mother cell and its bud (pedogamy) or between two different cells has also been identified (Kucsera et al., 2000).

P. rhodozyma grows in different carbon substrates (Kanwugu et al., 2020) and can produce different carotenoids like N-ASX, phoenicoxanthin, hydroxyechinenone, echinenone, and β-carotene, with N-ASX being the main product (83–87% of total). The accumulation of these molecules gives the reddish color to the yeast colonies. The cell wall of the yeasts is rigid and remarkably thick, it represents 20–25% of the dry weight of the cell, and it is in contact with the extracellular medium. The primary structural components of that wall are polysaccharides, principally glucans and mannans, and a minor percentage of chitin and proteins. On the other hand, the plasma membrane of yeasts is a thin lipid bilayer with proteins that control the material exchange in the cell (Stewart and Stewart, 2017). N-ASX production by *P. rhodozyma* involves a growth phase, in which high cellular density is reached, and a maturation phase, where a stress condition may contribute to N-ASX formation (Schewe et al., 2017; Zhang et al., 2020b). It has been shown that stress triggers relevant structural changes in the yeast cell envelop system and reduces cell membrane fluidity (Qiu et al., 2019), which complicates obtaining the N-ASX produced by *P. rhodozyma*. Although, at this point, N-ASX produced by *P. rhodozyma* is destined for the aquaculture market, this yeast may eventually be able to compete with the *H. pluvialis* for N-ASX production for other applications.

Paracoccus carotinifaciens

The species *carotinifaciens*, *haeundaensis*, and *marcusii* from the genus *Paracoccus* have been reported in the production of N-ASX; especially, *P. carotinifaciens* is gaining significant relevance since its dry cell biomass has been approved by the United State FDA and the EFSA as a coloring agent in salmon and trout aquaculture (Katsumata et al., 2014). *P. carotinifaciens* (class Alphaproteobacteria; order Rhodobacterales; family Rhodobacteraceae) is a rod-shaped, nonspore-forming, motile by peritrichous flagella, Gram-negative, aerobic bacteria found in soil. The cells can be 0.30–0.75 µm in diameter and 1.0–5.0 µm in length and assemble round, smooth, orange colonies. The optimum growth of this bacteria is 28 °C and pH 6.5–7.5. It uses ammonium but not nitrate as nitrogen source and a wide variety of compounds like glucose, mannose, maltose, xylose, galactose, sucrose, lactose, and glycerol (Tsubokura et al., 1999). The cell envelope in Gram-negative bacteria consists of an outer membrane in contact with the external environment and an inner membrane, in contact with the intracellular material. The cell wall is located between these two membranes and is composed of peptidoglycan, a copolymer formed by N-acetyl-glucosamine and N-acetyl muramic acid. Unlike Gram-positive bacteria, the peptidoglycan content is lower in Gram-negative bacteria, making them less resistant to cell breakdown (Silhavy et al., 2010; Sutterlin et al., 2016; Starke et al., 2019). *P. carotinifaciens* produces a wide range of carotenoids, N-ASX being 55–60% of the total carotenoids of the cell. The N-ASX produced by this bacterium is nonesterified in the 3S, 3'S form, just like in wild Atlantic or Coho salmon (Katsumata et al., 2014). The N-ASX produced by this bacterium may be a potential candidate for human consumption since this molecule is the same as that produced by *H. pluvialis* (3S, 3'S form), which has been tested in many clinical trials (Davinelli et al., 2018; Giannaccare et al., 2020).

Nonnative Producing Microorganisms

Production of N-ASX in nonnative host microorganisms has been carried out through genetic engineering or metabolic engineering techniques. Although these strategies have allowed a considerable increase in the product concentration (Zhang et al., 2020a), N-ASX from these microorganisms is not currently allowed for human consumption or fish feed supplementation. There is still little consumer acceptance for products from a genetically modified organism (GMO); additionally, the regulatory approval can take a long time and may vary in different countries. On the other hand, N-ASX produced in GMOs has shown no toxic effects and abnormalities in animal models and has been effective in inhibiting metastasis of melanoma cells in a rodent model (Tseng et al., 2020). Simultaneously, many commercial pharmaceutical products are produced in GMOs, which means that this strategy could be essential in the future of the biotechnological production of N-ASX. The use of GMOs to produce N-ASX offers many advantages in biotechnological processes. These microorganisms are easy handed, characterized by single-stage N-ASX production, and considered GRAS and grow fast in single substrates, and the

fermentation process is simple. Besides, they reach high N-ASX productivities, their genomes are easy to manipulate, and they have been used to produce commercial recombinant products, among them pharmaceutical products (Tripathi, 2016; Zhang et al., 2020a). Bacteria and yeasts have been genetically modified for N-ASX production; some of these microorganisms and their characteristics are shown in **Table 3**.

THE DOWNSTREAM PROCESS IN N-ASX PRODUCTION

When selecting the recovery and purification processes, the aspects that must be considered are the location of the product (intracellular or extracellular), the concentration of the product and impurities in the fermentation broth, the physical and chemical properties of the molecule, the intended use of the product (purity), and the market price (Stanbury et al., 1995). An efficient downstream process in N-ASX production may increase productivity, decrease production costs, and make a competitive process against S-ASX production, even for aquaculture purposes. Many studies focus on optimization of N-ASX biosynthesis by the microorganism (strain selection, growth conditions, culture media, and genetic manipulation), while the downstream processes is an area that urgently needs improvements for N-ASX production to be competitive. A general downstream process workflow for N-ASX production may include: 1) cell recovery, 2) cell disruption, 3) drying, 4) extraction, 5) purification, and 6) formulation (**Figure 2**). As we discuss below, some strategies can be combined to avoid some stages of the workflow and reduce production costs.

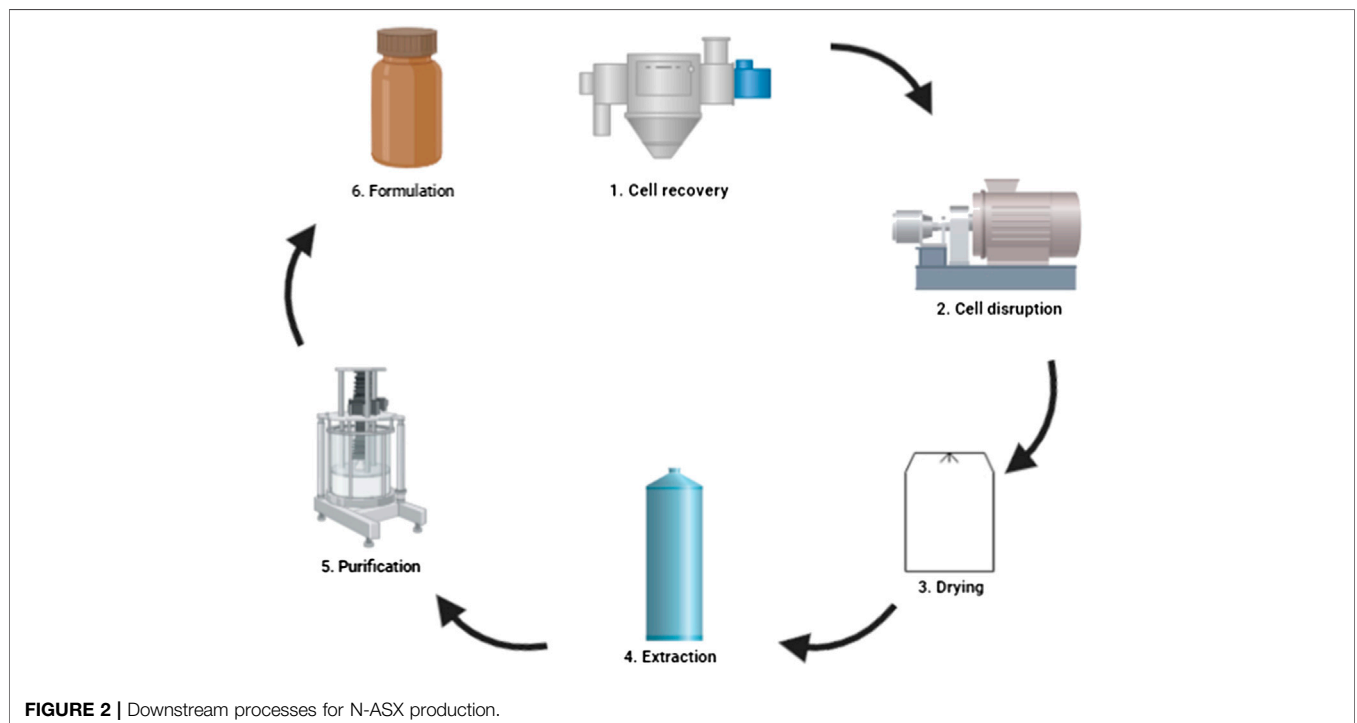
Cell Recovery

Once the N-ASX has been produced by a biotechnological process in a bioreactor, the first step of the downstream process involves cell recovery. Because the product is intracellular, the recovered cells represent the total N-ASX synthesized by the microorganism. In this way, a suitable recovery method that ensures the most significant number of cells and reduces the associated costs with cell dewatering must be selected.

The microbial cells can be harvested mainly by gravity sedimentation, flocculation, flotation, membrane filtration, and centrifugation. Gravity sedimentation is an economical option, but this process strongly depends on the particle size (biomass), cellular concentration, differences between the cell and the liquid densities, and the cell's ability to aggregate (Panis and Carreon, 2016; Liu et al., 2017). Flocculation refers to the aggregation of unstable and small particles through surface charge neutralization, electrostatic patching, or bridging after the addition of flocculants, which accelerate solids sedimentation (Matter et al., 2019). In the case of pharmaceutical products, flocculants are not recommended as they cause product contamination and make future purification difficult (Liu et al., 2017). The flocculation process is influenced by cell surface properties, cell concentration, pH, ionic strength, type and dosage of flocculants, and mixing process (Branyikova et al.,

TABLE 2 | Top ten ranked N-ASX-producing companies over the world according to Venture radar (2020).

Product name	Company	Country	Process characteristics	References
BioAstin and soft gel capsules	Cyanotech Corporation	United States	Cultivation of <i>H. pluvialis</i> in photobioreactor and open ponds. The cells are harvested and milled and the carotenoid was extracted by solvent-free supercritical CO ₂	Cyanotech Corporation (2020)
A wide variety of products for use in clinics: AstaReal ACT 2, Astarism, AstaCare, and AstaMed MYO	AstaReal	United States	Cultivation of <i>H. pluvialis</i> in an indoor bioreactor system	Astareal (2020)
AstaPure	Algatech International	Israel	Cultivation of <i>H. pluvialis</i> in a 600 km glass tubes' photobioreactor and carotenoid extraction by solvent-free supercritical CO ₂	Algatech International (2020)
Tablets, powder, beadlets, softgels, and oleoresin	Algatech International	Israel	Cultivation of <i>H. pluvialis</i> in a 600 km glass tubes' photobioreactor and carotenoid extraction by solvent-free supercritical CO ₂	Algatech International (2020)
AstaZine	Beijing Ginkgo Group	Switzerland	Cultivation of <i>H. pluvialis</i> in photobioreactor system and carotenoid extraction by solvent-free supercritical CO ₂ or by solvent extraction with ethanol	Beijing Ginkgo Group (2020)
Oleoresin, powder, and beadlets	Beijing Ginkgo Group	Switzerland	Cultivation of <i>H. pluvialis</i> in photobioreactor system and carotenoid extraction by solvent-free supercritical CO ₂ or by solvent extraction with ethanol	Beijing Ginkgo Group (2020)
Supplier of N-AXS and dried algal biomass rich in N-ASX	Noah's Inc.	United States	N-ASX produced from the cultivation of <i>H. pluvialis</i>	Noah's Inc. (2020)
Supplier of dried, fresh, concentrated N-AXS or live culture of <i>H. pluvialis</i>	MicroA	Norway	Cultivation of <i>H. pluvialis</i> in photobioreactor system	MicroA (2020)
A wide variety of products	KeyNatura	Iceland	N-ASX produced from the cultivation of <i>H. pluvialis</i>	Keynatura (2020)
AstaSkin, AstaEnergy, AstaEye, algae omega-3, and Icelandic Astaxanthin	KeyNatura	Iceland	N-ASX produced from the cultivation of <i>H. pluvialis</i>	Keynatura (2020)
Supplier of Astalif 5 and Astalif 10	Algalif	Iceland	Cultivation of <i>H. pluvialis</i> in a three-phase photobioreactor cultivation system. The cells are harvested and cracked and the carotenoid is extracted by solvent-free supercritical CO ₂	Algalif (2020)
Oleoresins soft gelatin capsules	Algalif	Iceland	Cultivation of <i>H. pluvialis</i> in a three-phase photobioreactor cultivation system. The cells are harvested and cracked and the carotenoid is extracted by solvent-free supercritical CO ₂	Algalif (2020)
A wide variety of products	Valens international	Unknown	Cultivation of <i>H. pluvialis</i> in closed greenhouse systems and open ponds. The cells are harvested, and the carotenoid was extracted by solvent-free supercritical CO ₂	Valensa International (2020)
Flex Pro MD, Flex Pro ES, Zanthin, and Eye Pro Carmis	Valens international	Unknown	Cultivation of <i>H. pluvialis</i> in closed greenhouse systems and open ponds. The cells are harvested, and the carotenoid was extracted by solvent-free supercritical CO ₂	Valensa International (2020)
Oleoresin, beadlets, and softgels	Valens international	Unknown	Cultivation of <i>H. pluvialis</i> in closed greenhouse systems and open ponds. The cells are harvested, and the carotenoid was extracted by solvent-free supercritical CO ₂	Valensa International (2020)
NeoAlgae Spirulina + Astaxantina and AHserum	NeoAlgae microseaweeds products SL	Spain	N-ASX produced from the cultivation of <i>H. pluvialis</i>	NeoAlgae Micro Seaweeds Products (2020)
Capsules and facial cream	NeoAlgae microseaweeds products SL	Spain	N-ASX produced from the cultivation of <i>H. pluvialis</i>	NeoAlgae Micro Seaweeds Products (2020)



2018). Flotation is another operation that can be used for cell harvesting. In this process, gas bubbles are supplied to the broth, causing cells to transport to the liquid surface. The process mechanism depends on the formation of stable microorganism-bubble complexes, and the difference in density between these complexes and the liquid causes the flotation of biomass and foam development. This microorganism-enriched foam can then be removed with a skimmer (Gulden et al., 2020). In this technique, microbial removal depends on the recycling rate, air tank pressure, hydraulic retention time, and particle floating rate, while the concentration of the produced slurry depends on skimmer velocity and relative positions towards the surface of the liquid (Barros et al., 2015). Filtration is a size-exclusion method in which cells pass through a suitable membrane under high pressure to obtain a thick paste of biomass. This process is beneficial and scalable for cell harvesting when problems in membrane blocking can be minimized or prevented; however, it can be expensive (Matter et al., 2019). In tangential flow filtration, the medium flows tangentially across a membrane, and the retentate is recirculated. The cells are kept in suspension, thus avoiding blockage of the membrane (Barros et al., 2015). Centrifugation involves separating liquids from solids of different densities based on a mechanical, gravitational force, accelerating the sedimentation of the highest density components. However, due to the intensive energy requirement and high investment cost, this method is recommended only for high-value products like N-ASX (Stanbury et al., 1995; Matter et al., 2019).

In the case of *H. pluvialis*, cells are relatively small (10–60 μm in diameter), its cellular concentration only reaches about 0.5% dry weight, the densities of the cultivation medium and the microalgae cells are similar and are negatively charged, which results in a stable dispersed state of the algal suspension that makes sedimentation challenging (Panis and Carreon, 2016; Liu et al., 2017). On the other hand, Orona-Navar et al. (2017) reported that *H. pluvialis* synthesizes an increased number of polysaccharides and carbohydrates during the aplanospore phase (accumulation of N-ASX), which results in the formation of aggregates and rapid sedimentation. Furthermore, the N-ASX accumulation resulted in a growth in cell size and increasing the settling velocity of *H. pluvialis* (Baroni et al., 2019). The cultures of N-ASX producing bacteria and yeasts (native or nonnative) reach higher cell densities in much less time than algae cultures, increasing the settling rate of the cells and facilitating biomass harvesting (Zhang et al., 2020b). When using yeast for N-ASX production, it is highly recommended to select a flocculating yeast strain. Some yeast strains of *S. cerevisiae*, *K. marxianus*, and *Y. lipolytica* can adhere to other cells and flocculate, which simplifies the recovery of the cells and reduces the production costs by avoiding the use of costly operations like centrifugation or filtration (Dasgupta et al., 2017; Di Gianvito et al., 2017; Louhasakul et al., 2019).

Gravity sedimentation is a cost-effective and energy-efficient technique; nevertheless, it progresses at a slow rate, and typically a complementary operation must be used. For most applications, *H. pluvialis* harvesting generally comprises a two-step concentration involving gravity sedimentation and

centrifugation (Barros et al., 2015; Zgheib et al., 2018; Li et al., 2020b). Centrifugation is a highly efficient method for biomass recovery (close to 100%); nonetheless, it represents a high investment cost and is high-energy consuming, which increases the production costs (Khoo et al., 2019b). The energy consumption of 1 kWh/m³ has been calculated in a disc-stack centrifuge to obtain 15–25% final slurry concentration of *H. pluvialis* (Panis and Carreon, 2016).

Najjar and Abu-Shamleh (2020) mentioned that centrifugation alone is not suitable for microalgae harvesting. They suggested that flocculation, as a primary preconcentration step, can increase the energy output significantly, thus reducing the operation cost of the separation process. Putri et al. (2020) used aluminum sulfate as flocculant at a concentration range of 0.25–2.0 g/L in *H. pluvialis* cell harvesting. The highest flocculation efficiency of 94.5% was obtained at 1 g/L of aluminum sulfate with a pH value of 3.69. This efficiency is comparable with the 95% recovery obtained by Panis and Carreon (2016) using centrifugation in the separation of *H. pluvialis* cells. Although the addition of flocculants is not recommended in pharmaceutical applications, the authors stated that aluminum sulfate is nontoxic, and it is used in vaccine making and as a food additive. The flocculation-based process is a simple, efficient, scalable, and low-cost technique that may contribute to the one-step cell concentration process (Matter et al., 2019). Najjar and Abu-Shamleh (2020) mentioned that the energy cost for centrifugation and filtration is more than USD 2.00/kg oil in microalgae, while the cost for flocculation is USD 0.25/kg oil. However, flocculation has not yet been sufficiently explored in *H. pluvialis* cell harvesting to draw concrete conclusions about its superiority.

Electroflocculation is an up-and-coming technique for cell harvesting based on the *in situ* generation of flocculants. The use of aluminum electrode plates during electrolysis produces a metal flocculant that contributes to cell flocculation due to charge neutralization, which creates the sorption affinity for negatively charged cells. Electroflocculation has been reported as a low-cost, high-efficient, and uncomplicated method. Although this technique has not been explored in the N-ASX production, recent microalgae harvesting reports suggest its usefulness in this biotechnological process. For example, Shi et al. (2017) applied current densities of 22.2, 44.4, and 66.7 A/m² in *Chlorella vulgaris* harvesting and reached a maximum harvesting efficiency of 98% for all treatments. Under higher current densities, shorter electrolysis time was needed to reach the maximum microalgae harvesting. Interestingly, the energy consumption for microalgae harvesting (higher than 80%) was 0.87×10^{-4} , 2.22×10^{-4} , and 2.94×10^{-4} kWh/g biomass for 22.2, 44.4, and 66.7 A/m², respectively.

Hydrocyclone is a low capital cost centrifuge; it can handle large quantities of slurry and may be an option to substitute costly equipment like a disc-stack centrifuge and multichamber centrifuge (Najjar and Abu-Shamleh, 2020). Cilliers and Harrison, (2019) harvested cells of a flocculating strain of *S. cerevisiae* using a hydrocyclone. The principal variables considered in this study were yeast concentration in a range of

TABLE 3 | Main genetically modified microorganisms (GMO) for N-ASX production.

Microorganism	Name	Characteristics	References
Bacterium	<i>Escherichia coli</i>	Gram-negative bacterium; the first GMO for N-ASX production; the highest concentration and yield of the carotenoid (native and nonnative microorganisms); producing pure 3S, 3'S N-ASX	Zhang et al. (2018); Zhang et al. (2020a)
Cyanobacterium	<i>Synechococcus</i> sp.	Photoautotrophic microorganism; naturally synthesized carotenoids	Hasunuma et al. (2019)
Bacterium	<i>Corynebacterium glutamicum</i>	Gram-positive bacterium; used for amino acid large-scale production; naturally synthesized carotenoids	Henke et al. (2016); Henke and Wendisch (2019)
Yeast	<i>Saccharomyces cerevisiae</i>	Widely used in traditional fermentations and new biotechnology; well accepted in food products; high fermentation capacity; genetically well characterized	Zhou et al. (2017); Jin et al. (2018)
Yeast	<i>Kluyveromyces marxianus</i>	Thermotolerant microorganism; producing aromatic alcohols used in cosmetics, fragrance, and food industries; present in probiotics; using lactose from whey waste to produce value-added products; producing 3S, 3'S N-ASX	Lane and Morrissey (2010); Tseng et al. (2020)
Yeast	<i>Yarrowia lipolytica</i>	Natural producer of carotenoid precursors; producing extracellular N-ASX; using lipids as the carbon source to produce N-ASX; the higher storage capacity of N-ASX over <i>E. coli</i> , <i>S. cerevisiae</i> , and <i>K. marxianus</i>	Li et al. (2020a); Zhang et al. (2020a)

2.0–14 g dry cell weight/L and yeast floc size, where 90% of the flocs were smaller than approximately 20, 100, and 200 μm distributions. It was observed that recovery was maximized with an increase in floc size and decrease in yeast concentration; the highest recovery (85%) was obtained at 2.0 g dry cell weight/L and 200 μm floc size.

Flotation is another alternative for cell harvesting that has very promising energy-saving characteristics. Implementing this economic process in N-ASX recovery can be beneficial in the downstream processes (Sankaran et al., 2018; Alhattab et al., 2019). One of the various flotation techniques is dissolved air flotation, where at very high pressures, gas bubbles are produced in size range of 10–30 μm . This method is characterized by microbubbles and high turbulence that increase the probability of flotation, especially for hydrophobic substances and fine particles such as microorganisms. Thus, dissolved air flotation is an appropriate option in N-ASX downstream processes application. Gulden et al. (2020) compared the specific product related to the cost of flotation (F), centrifugation (C), and cross-flow filtration (CFF). Based on the energy demand, maintenance, installation capital, and additives, the expected product cost calculated for *S. cerevisiae* cell recovery was about 6, 10, and 12 €/t product for F, C, and CFF, respectively. The authors studied the effect of operational parameters and found that the flotation rate of the yeast was improved by increasing the recycle flow rate and saturation pressure, whereas increasing the gas flow rate improved the flotation rate and the energy efficiency of the process.

Some cultivation strategies can be adopted to decrease production costs in *H. pluvialis* cell recovery. Do et al. (2019) produced N-ASX from *H. pluvialis* in a Twin-Layer Porous Substrate Photobioreactor (TL-PSBR), where biomass harvest is less complicated. This system includes a substrate layer (paper) in which a suspension of the algae is immobilized and through which the substrate is recirculated. The algae grow over the paper, and at the end of the algae cultivation, the paper is removed and dried with no centrifugation or filtration to remove excess water. After cell cultivation, the substrate layer was harvested and dried for 2 h at 105 °C and the highest biomass productivity was 8.7 g/m²d.

Cell Disruption and N-ASX Extraction

Cell disruption and N-ASX extraction are near related operations since the amount of the extracted carotenoid depends on the efficiency of the selected cell disruption method. Many technologies can be carried out in parallel to make the downstream processes faster and more efficient. **Table 4** shows recent reports about cell disruption and N-ASX extraction. These methodologies are discussed below.

Mechanical and Nonmechanical Cell Disruption Methods

Once the cells have been harvested, the next step is critical and encompasses the N-ASX release from producing microorganisms. Since N-ASX is an intracellular product, the breakdown of microalga, Gram-negative bacteria, and yeast cells needs to be carried out by different methods. The selected cell disruption method must be efficient and must break the largest number of cells possible since the more the cells are broken, the higher the amount of the product can be extracted, with a linear correlation between N-ASX recovery and the degree of cell disruption (Praveenkumar et al., 2020). The principal cell disruption techniques are bead milling, ultrasonication, osmotic shock, high-pressure homogenization, freeze-thawing, enzymatic lysis, and chemical lysis; they can be applied individually or in various combinations. Mechanical methods like bead milling and high-pressure homogenization may guarantee a high-yield N-ASX recovery; however, there is high-energy consumption associated with these techniques. These methods produce cell debris and have extended processing times, and the degradation may result in excessive heating.

It has been reported that bead milling and high-pressure homogenization are mostly used to disrupt large-scale processes for *H. pluvialis* cells (Panis and Carreon, 2016; Zgheib et al., 2018; Li et al., 2020b). In bead milling, the main operating parameters to take into account are rotational speed, time intervals, and the biomass/bead ratio (Irshad et al., 2019), while for high-pressure homogenization they are pressure and passage number (Praveenkumar et al., 2020). Chen et al. (2019) optimized the operating conditions of bead milling for the

disruption of *P. rhodozyma* cells. The cell disruption was carried out at yeast concentrations of 10, 20, 30, and 40 g/L, the temperature of 22, 30, 40, and 50 °C, and bead amount of 100, 200, 250, and 300 g/L. The maximum extraction yield (98%) was achieved at the cell concentration of 20 g/L, 40 °C, and 300 g beads/L in 5 h. Molino et al. (2018b) reported that the recovery of N-ASX from *H. pluvialis* cells was 86.4% when using bead milling, while the recovery was less than 1% without cell breakdown. On the other hand, Praveenkumar et al. (2020) applied a high-pressure homogenization method to disrupt *H. pluvialis* cysts in one to three passages and three different pressures (10,000, 20,000, and 30,000 psi). The cell disruption efficiency did not improve when increasing the pressure intensity, but the number of passages significantly improved this parameter. Since the maximum percentage of disrupted cells was about 91% for 3 passages and the three pressures applied, the recommended pressure was 10,000 psi.

Although bead milling and high-pressure homogenization have shown high efficiency for cell disruption, nonmechanical methods are highly relevant since they may be more energy-efficient and represent a more economical alternative. Although enzymes are cost-intensive, they have been used in the cell disruption of *H. pluvialis* and *P. rhodozyma* due to their high specificity to the cell wall substrates of these microorganisms. A combination of various types of enzymes, instead of just one type, acts simultaneously and leads to a more effective N-ASX release. It has been reported that the presence of β -1,3-glucanase, protease, cellulose, and pectinase gives high cell disruption and high N-ASX extraction yields in *H. pluvialis* and *P. rhodozyma* (Harith et al., 2020; Ye et al., 2020).

Ionic liquids (ILs) are recently being used in N-ASX extraction, and they are considered as a real alternative to mechanical cell disruption methods (Choi et al., 2019; Liu et al., 2019b; Tan et al., 2020). ILs are salts comprised of relatively large asymmetric organic cations coupled with smaller inorganic or organic anions. Due to several properties such as high hydrolysis activity, remarkable solubilizing capacity for various organic and inorganic compounds, high conductivity, low volatility, low viscosity, and high thermal and chemical stabilities, ILs are considered “green” solvents. Furthermore, ILs can be specifically formulated to suit precise conditions and to solubilize particular compounds by altering their functional groups. They can be recycled for several cycles without any previous treatment and a reduction in efficiency. When using ILs, the concentration, temperature, processing time, and type of ILs are important parameters to consider. Choi et al. (2019) evaluated ten types of 1-ethyl-3-methylimidazolium [Emim]-based ILs at different temperatures, concentrations, and processing times to disrupt *H. pluvialis* cell walls [Emim] HSO₄, [Emim] CH₃SO₃, and [Emim] (CF₃SO₂)₂N which were selected and their optimized concentration for cell disruption and N-ASX extraction of about 100% were 26.7% (v/v), 20.0% (v/v), and 26.7% (v/v), respectively. The optimized temperature and processing times were 30 °C and 60 min for the three [Emim]-based ILs. At the same time, ILs have shown superior performance against other nonmechanical cell disruption methods. Ye et al. (2020) compared three types of ILs,

cellulase, pectinase, and cellulase/pectinase in proportions 1:1, 1:2, and 2:1, and HCl 2, 4, and 6 M for the breakdown of *H. pluvialis* cells. The highest N-ASX extraction (86.71%) was achieved with 1-butyl-3-Methylimidazolium chloride [Bmim] Cl, while pectinase/cellulase at ratio 1:1 reached 71.0%, and HCl at 4 M reached 80.52%. Electron microscopy and transmission electron microscopy showed that all treatments hydrolyzed cellulase, hemicellulose, and lignin in the cell wall.

Extraction With Conventional Solvents

During N-ASX extraction, the largest amount of the carotenoid is obtained by using a two-step process. The first step involves cell disruption, whereas the second step involves organic solvent extraction. This strategy guarantees a high N-ASX recovery. Irshad et al. (2019) mention that if cyst cells of *H. pluvialis* are entirely disrupted, medium-to-high recovery of N-ASX is obtained 8–23 mg/g in the solvent extraction process. However, the two-step approaches are high-energy and time-consuming. Simultaneous cell disruption and extraction have been introduced as a new one-step approach that can be a cheaper alternative for N-ASX extraction. In solvent extraction, the solvent must be carefully chosen as it can be flammable, volatile, and often toxic. The use of green solvent, produced from renewable resources or environmentally benign, is strongly recommended (Saini and Keum, 2018). In the case of N-ASX extraction, ethanol, acetone, hexane, isopropyl alcohol, dichloromethane, and dimethyl sulfoxide (DMSO) are the most used, and the FDA classifies them as low risk to human health; they are approved, under certain limits, for their use in pharmaceuticals (FDA, 2020). The solvents' effectiveness is related to their polarity and their capacity to permeate through the cell wall and cell membrane (Harith et al., 2019). Harith et al. (2019) compared ethanol, methanol, DMSO, and acetone, combined with simple glass beads disruption, in N-ASX extraction from *P. rhodozyma*. Although N-ASX was shown to have a lower solubility in acetone than in DMSO, the extraction efficiency was 95% and 40%, respectively. The authors mentioned that N-ASX exhibited superior solubility in some of the solvents; however, these solvents could not permeate through the cell wall and cell membrane and failed to extract the carotenoid attached to these structures. Irshad et al. (2019) carried out the N-ASX extraction from *H. pluvialis* using a ball mill grinding in the presence of different solvents at rotational speeds of 100–200 rpm, time intervals of 5–30 min, and temperature of 25 °C. N-ASX recovery of >98% was achieved at 200 rpm, 30 min, with ethanol, acetone, and ethyl acetate. The extraction time for the one-step process (simultaneous wet grinding and solvent extraction) was 30 min, while the two-step process (dried biomass grinding and subsequent solvent extracting) took 60 min for maximum recovery of the carotenoid.

Since Gram-negative cells are more fragile than yeast and alga cells, the use of a solvent can be enough to disrupt *E. coli* and *P. carotinifaciens* and extract N-ASX in a single step. In the case of *E. coli*, the use of acetone at 55 °C (Lu et al., 2017; Li and Huang, 2018; Park et al., 2018) is sufficient for cell disruption and extraction, whereas ethanol at 60 °C is widely used for extraction from *P. carotinifaciens* cells (Honda et al., 2020a). Currently, there is little information about studies on N-ASX

solvent extraction from bacteria, so further research should be generated in this area. It is of great interest to study different types of solvent, concentration, processing time, and simultaneous use of solvents with other techniques, and optimize these parameters. The use of conventional solvent extraction offers a simple approach; however, low efficiency (extraction yield), selectivity (purity), high solvent consumption, and long treatment times show the need to look for new extraction alternatives (Poojary et al., 2016).

Nonconventional Extraction Methods

Alternatively to conventional solvent extraction, pressurized liquid extraction (PLE) is a technique where the solvents are applied at high temperatures and high pressures. The solvents are maintained in liquid states and penetrate easier into the cellular matrix, with that the solubility and extraction of N-ASX significantly increased. High temperatures may affect the thermolabile carotenoids' stability, so an increase in temperature must be done with great care (Gallego et al., 2019). Gallego et al. (2020) applied PLE in the presence of sea sand and pure ethanol at different temperatures, 10 MPa, and 20 min. The maximum concentration of total carotenoids 91.57 mg/g extract was achieved at 50 °C, while at temperatures higher than 100 °C, degradation of carotenoids was observed. Xiao et al. (2009) compared the use of HCl at 0.5 M under autoclave conditions (121 °C) for 5 min with HCl at 5M, 30 °C for 30 min in *P. rhodozyma* cells. The use of autoclave conditions led to a superior N-ASX extractability (84.8%) in much less time compared to high acid concentration treatment (only 72.5% extractability). Besides, N-ASX degradation was decreased by the short-time exposure to autoclave high temperatures.

In supercritical fluid extraction (SFE), the gas and liquid states of fluid cannot be differentiated under specific pressure and temperature conditions (critical points). A supercritical fluid presents high permeability, diffusion, and penetrates rapidly into the pores of complex matrices. SC-CO₂ is a relatively new technology applied in N-ASX extraction, and it has become the most used technique in the large-scale production of this carotenoid (Panis and Carreon, 2016; Li et al., 2020b). SC-CO₂ diffuses more efficiently through the cells than conventional organic solvents. It operates under mild temperatures (40–60 °C), which avoids the degradation of the compound and results in a solvent-free final product, easily separated from the N-ASX in the depressurizing process. SC-CO₂ is not flammable and is regarded as a safe solvent (Molino et al., 2020). Since SC-CO₂ is a nonpolar solvent, generally, a cosolvent is used to increase extraction efficiency due to the low solubility of N-ASX in nonpolar solvents. Ethanol is mostly used for this purpose in SC-CO₂. This alcohol is considered GRAS without any concentration limits (Molino et al., 2018a). When using SFE, the main operational conditions that affect the extract composition are temperature, pressure, type and concentration of cosolvent, solvent flow rate, and extraction time. The main limitation of SFE is the high cost of the equipment. Also, the high operational pressure (50 MPa or more) increases the operational cost (Reyes et al., 2014; Saini and Keum, 2018; Molino et al., 2020; Li et al.,

2020b). Molino et al. (2018a) evaluated the use of SC-CO₂ in *H. pluvialis* extraction under different parameters, namely, the extraction time, temperature (50, 65, and 80 °C), pressure (100, 400, and 550 bars), and ethanol as a cosolvent. Temperature and pressure significantly affected the carotenoid extraction, while ethanol concentration did not influence the N-ASX extraction. The highest N-ASX recovery 18.5 mg/g (92%) was reported at 65 °C, 550 bars for 20 min. Although molecule degradation occurred at 80 °C, the highest purity of 34% was found at this temperature at 440 bars, 80 min, without cosolvent. The use of SC-CO₂ was recently reported in *P. rhodozyma* by Harith et al. (2020). The authors studied the effect of the temperature, pressure, and ethanol concentration as a cosolvent in enzymatic pretreated cells and found that both pressure and cosolvent concentration significantly influenced N-ASX extraction. Although a prediction model for the extraction process was generated, the extraction efficiency only reached 22% (w/w) under the optimized conditions (76.8 °C, 360 bars, and 18% v/v ethanol concentration). The authors argued that enzymatic pretreatment with the commercial enzyme accelerase 1,500 (cellulase, hemicellulase, and β -glucanase activities) was not enough to disrupt the yeast cells since N-ASX extraction of ~90% has been reported in SC-CO₂ using a bead mill to disrupt *P. rhodozyma* cells before extraction (Lim et al., 2002). SC-CO₂ has also been reported in N-ASX producing bacteria by Chougale et al. (2016). These authors formulated a mathematical model, based on experimental data, to make a complete optimization of N-ASX extraction from *Paracoccus* sp. The parameters analyzed were pressure, temperature, particle size of biomass, time, type of cosolvent, and cosolvent concentration. Pressure and biomass particle size had a significant effect, and maximum recovery of 963.33 μ g/g was obtained under optimal conditions, while recovery of 317 μ g/g was obtained with conventional acetone extraction.

In ultrasound-assisted extraction (UAE), ultrasonication is combined with solvents or enzymes to enhance mass transfer and consequent disruption of the cell wall. This technique has increased N-ASX recovery in less time since the acoustic cavitation leads to cell rupture enhancing the mass transfer of extractants. It has been reported that the use of a probe (horn) sonicator instead of a bath sonicator generates a more intense and uniform vibration zone, increasing the effectiveness of the treatment. The main parameters to control when using UAE are the ultrasonic power, intensity, temperature, and sample-to-solvent ratio (Haque et al., 2016; Saini and Keum, 2018). Machado et al. (2016) extracted N-ASX from *H. pluvialis* cells using three commercial enzymes assisted by ultrasound. The enzymatic reaction conditions of Gucanex (β -1,3-glucanase and protease activities from *Trichoderma harzianum*), Driselase (β -1,3-glucanase and xylanase activities from *Basidiomycetes* sp.), and Lyticase (β -1,3-glucanase and protease activities from *Arthrobacter luteus*) were optimized. The highest extractability was 83.9 and 85.06% for unfrozen and frozen cells, respectively, using Glucanex lysis assisted with ultrasound, while 79.34 and 81.4% were achieved in unfrozen and frozen cells, respectively, without ultrasound. Urnau et al. (2018) reported a 99% (680 μ g/g) and 89.32% (614.25 μ g/g) carotenoid recovery in

P. rhodozyma using only DMSO and UAE acetone/methanol mixture, respectively. Although extraction with DMSO was superior, the processing time was considerably reduced by 45% with ultrasound.

Pulsed electric field (PEF)-assisted extraction is a promising technique that consists of the application of repetitive short high voltages pulses to induce permeabilization (electroporation) of biological material (plant, animal, or microbial cells). This technique requires low energy expenditure, easily scalable and nonthermal, and facilitates the purification of extracted compounds since it minimizes the release of cell debris. The pulse duration and electric field strength intensity are parameters that need to be optimized for each sample since they may change with a change in the electrical conductivity and texture of the sample (Saini and Keum, 2018; Aguilar-Machado et al. (2020)). It has been suggested that PEF also triggers some enzyme activity, which in turn accelerates the autolysis and the subsequent release of N-ASX. Aguilar-Machado et al. (2020) evaluated PEF treatment with electric field strength between 10 and 25 kV/cm at 0.5 Hz frequency in a wild-type and mutant *P. rhodozyma*. The PEF pretreatment was ineffective for N-ASX extraction with ethanol in the two yeast strains. However, 2.43 mg/g (dw) (70% extractability) of carotenoids was extracted after aqueous incubation for 12 h of PEF-treated mutant strain cells at 25 kV/cm. The extraction of N-ASX was mediated by esterase activity triggered by PEF during incubation since this enzyme activity was higher in PEF-treated mutant cells compared with no treated mutant cells.

In microwave-assisted extraction (MAE), high-frequency waves (300 MHz–300 GHz) are generated and cause dielectric heating in the sample, primarily by absorption of water and other energy polar compounds present in wet biomass, resulting in temperature increases in the intracellular liquids, subsequently causing the water to evaporate and exert pressure on the cell wall leading to cell disruption (Kapoor et al., 2018). MAE's main advantages are significantly decreased extraction time and a single-step process that combines cell disruption and compound extraction (Zhao et al., 2016; Fan et al., 2019). The irradiation power, irradiation time, and the number of cycles are the most important parameters when using this technology. Fan et al. (2019) used MAE with three protic ILs (PILs) to extract N-ASX from *H. pluvialis*. Ethanolammonium caproate (EAC) was selected, and the extraction efficiency of ~35 mg/g was obtained at 210 W, 5.2 mol/L EAC concentration, two extraction cycles (50 s each), and liquid-solid ratio of 20 g/g. The short extraction time made this strategy very relevant for large-scale implementation. Furthermore, the authors listed other PIL advantages: low cost, easy synthesis, and low toxicity. Zhu et al. (2010) selected the frequency of 416.5 MHz MAE in the presence of DMSO to break the cells of *P. rhodozyma* and extract N-ASX in ethanol. They reported a 22% yield increase upon optimization of cell breaking time, ethanol volume, and extraction time.

It has been reported that N-ASX Z-isomers exhibit higher bioavailability and antioxidant activity than the all-E-isomers. Thus, the obtention of Z-isomers is of great interest for pharmaceutical use. The conversion of all-E-isomers to

Z-isomers can be achieved by thermal treatment and different lighting and solvents (Yang et al., 2017). Besides, N-ASX Z-isomers are superbly soluble in ethanol than all-E-isomers (~700 times more), which further facilitate the extraction (Honda et al., 2018). Honda et al. (2020a) compared different electrophilic compounds to enhance the extraction and thermal Z-isomerization of all-E-carotenoids from dried cell powder of *P. carotinifaciens*. The N-ASX extraction increased 27% compared to the control when allyl isothiocyanate (isomerization catalyst), from the *Brassicaceae* plant family, was used at 100 mM along with ethanol at 60 °C for 1 h. Also, the presence of the catalyst increased the content of N-ASX Z-isomer by 125%. The use of allyl isothiocyanate represented an efficient and environmentally friendly method for the extraction and Z-isomerization of the carotenoid.

New fermentation strategies are being explored to obtain N-ASX without any pretreatment of the cell (cell recovery, cell disruption, and cell drying). In extractive fermentation, some compounds allow a selective and continuous extraction of the product from the live cells, which preserve their integrity and productivity (Samorì et al., 2019). Li et al. (2020a) reported the use of *Yarrowia lipolytica* in a fermentation process with 70% of sunflower oil as a sole carbon source. The yeast produced N-ASX at a 167 mg/L concentration and secreted continuously up to 48% of the total product to the medium. In a similar strategy, cells are “milked” and reused. Samorì et al. (2019) reported almond oil and cyclohexane for N-ASX extraction from *H. pluvialis*. The oil and the solvent were compatible with the alga and kept it alive; the cells maintain their photosynthetic activity and produced the carotenoid again when the fresh medium was added. Praveenkumar et al. (2015) reported using a gold nanoscalpel to make an incision in a cell of *H. pluvialis* and extract the N-ASX. The cell healed the wound and reaccumulated the carotenoid, even twice as fast as the control. In another approach, germination of the cyst cells of *H. pluvialis* was induced, and the zoospores containing N-ASX were released. The product was extracted directly from the broth using ethyl acetate (Bauer and Minceva, 2019).

Drying

Once the cells have been disrupted, a drying process is necessary to prevent unstable N-ASX degradation before extraction and extend the shelf life. An ideal strategy would be to extract the carotenoid from the wet material obtained after cell disruption, avoiding the drying operation. However, in large-scale production, this strategy may be challenging to achieve. On the other hand, the drying operation may be used in the formulation process, as will be seen later.

Despite high operation and investment costs, spray drying is the most used technique in large-scale N-ASX production (Li et al., 2011, Li et al. 2020b; Panis and Carreon, 2016; Zgheib et al., 2018; Dursun et al., 2020). In this process, a solution, suspension, or emulsion is converted to a dried powder in a single step by passing an atomized spray through a high-temperature gas (Ziaee et al., 2019). The inlet temperature and the drying chamber's outlet temperature are critical parameters in a spray drier equipment and must be carefully selected since degradation of

the N-ASX molecule may occur due to high temperatures. Raposo et al. (2012) dried a cell suspension of *H. pluvialis* by spray drying. The inlet/outlet temperatures tested were 220/120 °C, 220/80 °C, 180/110 °C, and 180/80 °C. The cells resisted the drying process, and N-ASX degradation occurred at different storage conditions. The authors found that powders with higher water content were more stable, and N-ASX degradation was slower. The drying process at 180 °C (inlet) and 110 °C (outlet) produced the most stable N-ASX powder, and less than 10% of the carotenoid was degraded after 9 weeks stored at -21 °C under nitrogen. Up to 80% of the molecule was degraded in nine weeks when the powder (obtained at 180 °C (inlet) and 80 °C (outlet)) was stored at 21 °C under N₂. Young et al. (2017) obtained powder of disrupted cells of *H. pluvialis* when operating a spray drier at temperatures 220 °C (inlet) and 140 °C (outlet), airflow 62 m³/h, pump flow 175 ml/h, and compressor pressure of 0.4–0.7 bars. Astatine, which is an indicator of oxidative damage in N-ASX, was not detected after drying. The stability of the products was measured at -20 and 4 °C in the presence or absence of N₂, and up to ~90% of N-ASX was retained over 16 weeks at -20 °C under N₂. Xiao et al. (2009) used spray drying in *P. rhodozyma* at temperatures of 170 °C (inlet) and 68 °C (outlet). When calculating the relative residual N-ASX percentage, 39% of the molecule was degraded by the drying process. An and Choi, (2003) studied the N-ASX degradation after *P. rhodozyma* cell disruption with 0.2 M HCl at 90 °C for 8 h and spray drying at temperatures of 150 °C (inlet) and 85 °C (outlet). The percentage of degradation was 11.2% when the cell disrupted suspension was adjusted to pH 3 with NaOH, while being 17% when the suspension was at pH 7. The authors suggested that a higher NaOH degrades the carotenoid to astacin when neutralizing the suspension to pH 7. The use of spray drying in N-ASX produced in *E. coli* has also been reported by Lin et al. (2017). In this study, the cells were spray-dried at 120–150 °C (inlet temperature); the carotenoid was extracted with ethanol and crystallized. The crystals contained 79.5% of N-ASX and showed no toxicity on a rodent model.

Alternatively, freeze-drying, also known as lyophilization, is a widely used technique in N-ASX-producing cells. It involves freezing the material at -20 °C for dehydration, so it is milder than the high temperature in spray drying (180–220 °C). However, freeze-drying is more expensive in large-scale industrial processes (Khoo et al., 2019b). Ahmed et al. (2015) compared freeze-drying with spray drying in *H. pluvialis* cells. The authors reported that, after the microalgae's drying process, the total N-ASX extracted with acetone was 6,454.9 µg/g in freeze-dried powder, while in spray-dried powder, it was only 4,578.6 µg/g. The powders obtained were stored under vacuum or in air at -20, 4, 20, and 37 °C, and it was showed that higher temperature and air storage accelerate N-ASX degradation. The freeze-dried N-ASX powder stored at -20 °C under vacuum showed higher stability, with only 12.3% degradation of the total carotenoid in 20 weeks of storage. Although freeze-drying is more expensive than spray drying, a cost-benefit analysis showed that freeze-drying was a better option. The freeze-drying process was used in N-ASX drying in GMO *S. cerevisiae*, but unfortunately, details of the process and its

effect on N-ASX stability were not reported (Ukibe et al., 2009). Freeze-drying has also been applied in *P. rhodozyma* by Gervasi et al. (2018) at -50 °C for 24 h and by Harith et al. (2020) at -20 °C for 24 h to preserve the cells before N-ASX extraction, but the effects of the process over the carotenoid stability were not explored.

Purification

N-ASX's bioactivity has been extensively tested in clinical trials, and its effectiveness makes it a compound with great potential as a nutraceutical or pharmaceutical product. When preparing this kind of product, the purity of the compound must be ensured. Once the extraction of N-ASX has been completed, purification is necessary due to impurities like other carotenoids, proteins, salts, esters, cell debris, antifoams, and solvents and other contaminants. In this section, we focused on the obtention of free N-ASX and the separation of this molecule and its esters. **Table 5** shows the details of recent studies about the purification processes in N-ASX.

The presence of esters in N-ASX is undesirable since they may interfere with the absorption of the compound. Mimoun-Benarroch et al. (2016) compared the bioavailability of S-ASX and N-ASX from the microalgae *H. pluvialis*, the bacteria *P. carotinifaciens*, and the yeast *P. rhodozyma* on Caco-2/TC7 colonic cells. In this study, S-ASX and N-ASX's absorption from bacteria and yeast was superior to N-ASX from microalgae. The authors mentioned that esterified N-ASX, produced only in the microalgae, needs to be hydrolyzed in the intestinal lumen before absorption, which causes a decrease in the process. Also, esters' presence also interferes with the chromatographic analysis/separation since esterified isomers cannot be distinguished unless the fatty acid chains of the N-ASX are removed. When removing ester groups, the carotenoid can be separated by chromatographic technics, and free N-ASX can be linked to water-soluble groups to form antioxidant drugs useful in pharmaceuticals (Su et al., 2018). The ester groups are generally removed by saponification using NaOH solution. However, this reaction may degrade the carotenoid molecules since the hydroxyl group in N-ASX can be dehydrogenated to form astacin. Incubation time, temperature, alkali concentration, and volume for partition and washing must be controlled when using saponification (Inbaraj et al., 2008; Mei et al., 2020). N-ASX esters can be hydrolyzed by the enzymolysis with lipase. The use of cholesterol esterase (EC 3.1.1.13) from *Pseudomonas fluorescens* is widely used by many *H. pluvialis*-producing companies to cleave carotenoid esters (Su et al., 2018). Su et al. (2018) reported complete enzymolysis with cholesterol esterase and saponification with NaOH of N-ASX. The maximum free N-ASX recovery was 89.5% when using the enzyme, and no semiastacin or astacin was observed, while the recovery was only 48.40% in the presence of alkali and semiastacin presence. The isomers all-*trans*, 9-*cis*, and 13-*cis* from the free carotenoid were present in 84, 12, and 4%, respectively.

Once the ester groups have been eliminated, free N-ASX and its isomers must be separated and purified. Several studies have reported using normal-phase chromatography and reverse-phase

TABLE 4 | Recent reports in N-ASX producing microorganisms cell disruption and N-ASX extraction methods.

Microorganism	Cell disruption and extraction method	Best conditions	N-ASX recovery	References
<i>H. pluvialis</i>	Simultaneous use of bead mill and different solvents	200 rpm, 30 min, 25 °C, using ethanol, acetone, or ethyl acetate	>98%	Irshad et al. (2019)
<i>H. pluvialis</i>	Use of high-pressure homogenization and subsequent solvent extraction	10,000 psi, 3 passages	15.2 mg/L	Praveenkumar et al. (2020)
<i>H. pluvialis</i>	Use of ILs solvents and subsequent solvent extraction	Three [Emim]-based ILs were selected [Emim] HSO ₄ at 26.7% (v/v), 30 °C, 60 min [Emim] CH ₃ SO ₃ at 20.0% (v/v), 30 °C, 60 min [Emim] (CF ₃ SO ₂) ₂ N at 26.7% (v/v), 30 °C, 60 min	100% for all cases	Choi et al. (2019)
<i>H. pluvialis</i>	Use of ILs solvents and subsequent solvent extraction	1 ml of 1-butyl-3-methylimidazolium chloride [bmim] Cl at 40% (v/w), 60 °C, 1 h	86.71%	Ye et al. (2020)
<i>H. pluvialis</i>	Simultaneous use of PLE with ethanol in the presence of sea sand	10.5 MPa, 20 min, 50 °C Carotenoids degradation occurs at 100, 150, and 200 °C	91.57 mg/g extract	Gallego et al. (2020)
<i>H. pluvialis</i>	Use of bead mill and subsequent PLE extraction with acetone	100 bars, 40 °C, 20 min	Total N-ASX: 17.281 mg/g biomass All-trans: 13.882 mg/g biomass 9-cis: 1.985 mg/g biomass 13-cis: 1.414 mg/g biomass	Molino et al. (2018a)
<i>H. pluvialis</i>	Use of bead mill and subsequent SC-CO ₂ extraction	CO ₂ 3.62 g/min, biomass 1.38 g, 65 °C, 550 bar, 20 min Ethanol did not improve extraction, and molecule degradation occurred at 80 °C	18.5 mg/g (92%)	Molino et al. (2018a)
<i>H. pluvialis</i>	Enzymatic lysis with a commercial enzyme, assisted by ultrasound	Glucanex (β-1,3-glucanase and protease activities from <i>Trichoderma harzianum</i>) at 55 °C, pH 4.5, 30 min, and initial β-1,3-glucanase activity of 0.6 U/mL. Ultrasound at 40 kHz	83.9 and 85.06% for unfrozen and frozen cells, respectively	Machado et al. (2016)
<i>H. pluvialis</i>	Use of PEF in methanol, UAE in methanol, HPMF/ethyl acetate, HCl/acetone, and ILs/methanol	PEF: 30 kV/cm, 30 min UAE: 600 W, 20 kHz, 30 min HPMF: 100 mpa, 3 passages HCl: 4 M, 70 °C, 1 h ILs: [Bmim] Cl 40%, 60 °C, 1 h	6.52% 11.79% ~80% ~80% ~80%	Liu et al. (2018c)
<i>H. pluvialis</i>	Use of PEF in the microalgae medium, bead mill, UAE, thermal treatment, freeze-thawing, and subsequent solvent extraction	PEF: 1 kV/cm (50 kJ/kg), 10 pulses of 5 ms, and aqueous incubation for 6 h Bead mill: 4,800 rpm UAE: 80% amplitude and 10 cycles during 10 s Thermal treatment: 70 °C, 1 h Freeze-thawing: fast freezing in liquid N ₂ and slow melting, 5 cycles	18.24 mg/g (96%) ~15.3 mg/g (~80%) ~15.3 mg/g (~80%) ~15.3 mg/g (~80%) ~15.3 mg/g (~80%)	Martínez et al. (2019)
<i>P. rhodozyma</i>	Use of MAE with PILs and subsequent ethanol extraction	EAC at 5.2 mol/L, 210 W, two extraction cycles for 50 s each, and the liquid-solid ratio of 20 g/g	~35 mg/g 97.2% purity	Fan et al. (2019)
<i>P. rhodozyma</i>	Use of MAE with DMSO and subsequent ethanol extraction	MAE 416.5 MHz, cell wall breaking time 2.60 min, and 3.0 ml of DMSO. Extraction at 50 °C for 15.1 min and 12.6 ml of ethanol	2.93 mg/L	Zhu et al. (2010)
<i>P. rhodozyma</i>	Use of bead mill without further extraction	20 g biomass/L, 40 °C, 300 g beads/L, and 5 h. The temperature of 50 °C caused N-ASX degradation over time. Full use of the extract without further extraction on N-ASX	98%	Chen et al. (2019)
<i>P. rhodozyma</i>	Chemical treatment, autoclave, spray drying, autolysis, and subsequent extraction with ethanol	HCl at 0.5 M under autoclave conditions (121 °C) for 5 min Treatment decrease degradation of the molecule significantly	85%	Xiao et al. (2009)
<i>P. rhodozyma</i>	Enzymatic cell disruption with two commercial enzymes and subsequent extraction with acetone	Glucanex (β-1,3-glucanase and protease activities from <i>Trichoderma harzianum</i>) at 30.8 °C, pH 4.6, and acetone for 10 min	94 and 116% (compared with a control procedure) for dry and wet cells, respectively	Harith et al. (2020)

(Continued on following page)

TABLE 4 | (Continued) Recent reports in N-ASX producing microorganisms cell disruption and N-ASX extraction methods.

Microorganism	Cell disruption and extraction method	Best conditions	N-ASX recovery	References
<i>P. rhodozyma</i>	Simultaneous use of bead mill and different solvents in dry cells	30 mg of cells, 0.3 g of glass beads, 200 rpm, 25 °C, and 1 ml of acetone for 10 min	95%	Harith et al. (2019)
<i>P. rhodozyma</i>	UAE in lactic acid and subsequent extraction in ethanol	Lactic acid 3 M, 15 min, 80 W, duty cycle of 60%	90%	Gogate and Nadar (2015)
<i>P. rhodozyma</i>	Use of UAE in acetone, maceration, abrasion, immersion in liquid N ₂ , sodium bicarbonate, DMSO, acids, commercial enzyme glucanex, and UAE with glucanex	UAE: 40 kHz, 10 min Maceration: 0.5 g of biomass and 0.5 g diatomaceous earth, 10 min; use of frozen cells Abrasion: 0.5 g biomass, 6 ml acetone, 1.1 g/ml glass pearls, 10 min agitation, and use of frozen cells Immersion liquid N ₂ : 0.5 g biomass, 6 ml acetone, and freezing in N ₂ Sodium bicarbonate: 0.5 g biomass, 18 ml of 0.1 M of sodium bicarbonate, 45 °C, 24 h, and use of frozen cells DMSO: 0.05 g biomass, 2 ml, 55 °C, 1 h, and use of frozen cells Hydrochloric acid 4 M: 0.05 g biomass, 7.5 ml, 5 min, and 35 °C, use of frozen cells Acetic acid 4 M: 0.05 g biomass, 7.5 ml, 5 min, and 35 °C Lactic acid 4 M: 0.05 g biomass, 7.5 ml, 5 min, and 35 °C Glucanex: 55 °C, pH 4.5, 30 min, and use of frozen cells UAE glucanex: 55 °C, pH 5.4, 30 min, and 40 kHz	88.38 µg/g (56.75%) 93.13 µg/g (59.81%) 75.42 µg/g (48.43%) 84.68 µg/g (54.38%) 36.96 µg/g (23.74%) 155.72 µg/g (100% control) 65.37 µg/g (41.98%) 30.55 µg/g (19.62%) 47.72 µg/g (30.65%) 156.96 µg/g (101.81%) 163.12 µg/g (104.76%)	Michelon et al. (2012)
<i>P. rhodozyma</i>	Use of SC-CO ₂ , high-pressure homogenization, bead mill, and UAE with solvents and enzyme	UAE β-glucanase: 35 °C, pH 7, 6 h. Ultrasound at 100% amplitude for 5 min	435.70 µg/g	Hasan et al. (2016)
<i>P. carotinifaciens</i>	Use of solvents and allyl isothiocyanate	Allyl isothiocyanate 100 mM with acetone, 60 °C, 1 h Allyl isothiocyanate 400 mM with ethanol, 60 °C, 1 h	97.9% N-ASX 40.0% Z-isomer N-ASX	Honda et al. (2020a)
<i>Paracoccus</i> sp NBRC 101723	SC-CO ₂ extraction and no cell disruption	40 °C, 350 bar, 60 min, 0.1 mm biomass particle size, and ethanol at 20% (v/w)	963.33 µg/g	Chougale et al. (2016)

HPMF, high-pressure microfluidization (higher operating pressure than high-pressure homogenization); ILS, ionic liquids; PLE, pressurized liquid extraction; PEF, pulsed electric field; MAE, microwave-assisted extraction; UAE, ultrasound-assisted extraction.

chromatography (C₁₈ column) for this purpose (Wang et al., 2008; Lafountain et al., 2013). Honda et al. (2020a) determined the conditions for separating N-ASX isomers from *P. carotinifaciens* by normal-phase HPLC. The best peaks separation was obtained in columns at 40 °C with silica gel and a mobile phase of hexane/ethyl acetate/acetone (70:20:10, v/v/v) at 1.2 ml/min. Yang et al. (2017) obtained N-ASX Z-isomers (9 and 13) from N-ASX all-E-isomers by using iodine-doped titanium dioxide (I-TiO₂) as the catalyst. The isomers were purified by HPLC on a Kinetex XB-C18 column (reverse-phase chromatography), and 22.7% and 16.9% of 9Z and 13Z isomers were obtained, respectively. The purity of 9Z isomer was 95.3%, while the purity of 13Z isomer was 91.8%. Besides,

Z-isomers were stable between pH 3.5 and 11.6 and in the presence of metal ions.

The purification process by chromatography is a scalable and straightforward operation. Sun et al. (2015) reported a general scheme for N-ASX purification from *H. pluvialis*. The crude extract of the carotenoid, obtained from the extraction process, was treated with 0.02 M NaOH at low temperatures. Liquid-liquid extraction (water/n-hexane) to remove alkali and chlorophyll impurities was carried out before the purification. The free N-ASX extract was passed through low-pressure silica gel (300–400 mesh) filled column chromatography (liquid-solid chromatography) for about 90 min, the carotenoid solution was crystallized, and the HPLC analysis showed a >96.5% purity. The

author mentioned that low-pressure column chromatography (LPCC) is a low-cost option and highly scalable in comparison to other chromatography techniques like high-speed countercurrent chromatography (HSCCC) and reverse-phase HPLC (RP-HPLC) used in this carotenoid purification. When using liquid-solid chromatography, a large load capacity of the solid phase needs to be ensured. Casella et al. (2020) evaluated the commercial activated carbon Darco G-60 (100–325 mesh) in N-ASX's purification from *H. pluvialis*. After accelerated solvent extraction at 100 bar, 67 °C with ethanol, the sample was subjected to chromatography in a column filled with 50, 100, and 200 mg of the commercial product at a flow rate of ~1.0 ml/min. The adsorption capacity of the activated carbon was high with a value of 22.6 mg/g, and the method represented an economical option for carotenoid purification.

HSCCC is a liquid-liquid method based on the partitioning of compounds between two immiscible liquid phases without solid support. The advantages of this technique are reversible adsorption, low risk of sample denaturation, large load capacity, and excellent sample recovery (Zhao et al., 2015; Du et al., 2016). Du et al. (2016) selected the two-phase solvent system used in HSCCC based on a partition coefficient value (*k*) for N-ASX in the range of 0.5–2.0. The system n-hexane-acetone-ethanol-water (1:1:1:1, v/v/v/v) was selected since it presented a *k* value of 1.408 and the best peak resolution for N-ASX. The crude extract, obtained by solvent extraction, was subjected to HSCC and the upper phase (n-hexane and a spot of acetone, ethanol, and water) was used as the mobile phase and the lower phase (acetone, ethanol, water, and a spot n-hexane) as the stationary phase. The separation yield was 20.6 mg/L at 92.0% purity, and by further one-step silica gel chromatography, the purity on N-ASX from *P. rhodozyma* was 99.0%. Some reports claim that esterified N-ASX has better or equal pharmacological properties than free N-ASX, which is also unstable and susceptible to oxidation (Rao et al., 2015; Régnier et al., 2015). Due to this, some studies have focused on the purification of the esterified N-ASX. Fábryová et al. (2020) purified five esterified N-ASX molecules by high-performance counterchromatography (HPCCC), where n-heptane and acetonitrile (ratio 5:5) were used as the mobile phase in the lower phase of a biphasic solvent system, and a final purification step was carried out using semipreparative HPLC. The yields of the purified compounds were between 1 and 12 mg, exhibiting purities over 98%. Moreover, the purified esterified N-ASX oleic acid monoester had a cytotoxic effect against human gastric cancer cells.

In supercritical antisolvent fractionation (SAF), the SC-CO₂ is in continuous contact with a polar liquid mixture (solvent + compounds extracted from a natural matrix) in a pressurized precipitation vessel. The supercritical fluid dissolves the polar solvent and the compounds soluble in the mixture SC-CO₂/solvent, selectively precipitating heavier or more polar compounds that will not be soluble in the fractionation medium (Gonzalez-Coloma et al., 2012). Gallego et al. (2020) reported this technique, coupled with two-dimensional liquid chromatography for *H. pluvialis* in N-ASX purification. The authors extracted N-ASX with pressurized ethanol, and after optimization of SAF parameters, an enriched extract with a

value of 103.3 mg/g N-ASX was obtained by using 30 MPa CO₂ pressure, 0.05 PLE extract/SC-CO₂ mass flow rate ratio, and 20% (v/v) of water in the feed solution. The use of normal-phase chromatography in the first dimension and reverse-phase chromatography in the second dimension allowed the separation of N-ASX monoester, diesters, and free N-ASX in just 25 min.

Formulation

The formulation of N-ASX is the final step in the obtention of the nutraceutical or pharmaceutical products. Significant efforts have been carried out to obtain stable formulations containing bioactive molecules of the carotenoid. Since N-ASX quickly decomposes when exposed to light, oxygen, and/or heat and is poorly soluble in water, there is a need to obtain a formulation that minimizes these problems. Many reports have focused on the encapsulation process to enhance N-ASX stability and bioavailability. This procedure may protect the carotenoid from the acidity of gastric juice while allowing its subsequent release in the intestinal fluids (Liu et al., 2018a). Encapsulation is a process for trapping solids, liquids, or gaseous materials within a continuous film coating from another substance, producing particles with different diameters (Ozkan et al., 2019). According to their size, the capsules can be classified into macrocapsules (~5,000 μm), microcapsules (1–5,000 μm), and nanocapsules (<1 μm); the last two are of interest for medical therapy. On the other hand, capsules can be divided into microcapsules and microspheres according to their shape and construction. Microcapsules are formed by an inner core (nuclei) that contains the active substance, covered with a layer of polymer, while in microspheres, the nucleus is dispersed and dissolved uniformly inside a polymer matrix (Santos-Sánchez et al., 2020).

Conventional encapsulating techniques used in N-ASX formulation include spray drying, lyophilization, solvent evaporation, coacervation, liposomes, and ionic gelation (Dorđević et al., 2014; Ozkan et al., 2019). In spray drying, the feed liquid may be a solution and emulsion or a suspension. Typical wall materials are polysaccharides and proteins. The characteristics of spray-dried powders are related to the factors discussed in Drying, as well as the sort of carrier agent and its concentration (Fatnassi et al., 2013). Freeze-drying has been used in many heat-sensitive antioxidants due to its low operating temperature; however, this technique characterizes by long process time (20 h or more), and the powders must be grounded after the drying process (Anwar and Kunz, 2011). In solvent evaporation, an emulsion of a polymer, active compound, organic solvent, and aqueous phase is prepared, and the solvent is removed by evaporation or liquid extraction. The particles are recovered and dried (Tiwari and Verma, 2011). Coacervation is a colloidal phenomenon that involves liquid-liquid phase separation of a single polymer (simple coacervation) or a mixture of two oppositely charged polymers (complex coacervation) in an aqueous solution triggered by physical forces, chemical interactions, or enzymatic cross-linker agents (Xiao et al., 2014). Liposomes are vesicles with single or multiple bilayers (principally phospholipids) with a hydrophilic head and hydrophobic tail groups. They are obtained by the dispersion of

TABLE 5 | Strategies for N-ASX purification.

Microorganism	Conditions for purification	Yield recovery or purity	References
<i>H. pluvialis</i>	Elimination of ester groups Cholesterol esterase from <i>Pseudomonas fluorescens</i> at 0.67 U/mL, 37 °C, darkness for 60 min NaOH at 0.021 M at 5 °C, darkness for 90 min	89.5% free N-ASX recovery, proportions of all- <i>trans</i> :9- <i>cis</i> :13- <i>cis</i> = 21:3:1 and no degradation occurred through reaction time 48.4% free N-ASX recovery, proportions of all- <i>trans</i> :9- <i>cis</i> :13- <i>cis</i> = 21:3:1 and degradation occurred through reaction time	Su et al. (2018)
<i>H. pluvialis</i>	Elimination of ester groups Lipase from <i>Aspergillus niger</i> at 5000 U/mL, 40 °C, pH 6.0. 200 min in a microchannel reactor to avoid the use of emulsifiers or solvents in the enzymatic reaction	75.4% hydrolysis rate, 18.8 mg/L free N-ASX, 0.450 g/h productivity	Mei et al. (2020)
<i>H. pluvialis</i>	Purification by LPCC Saponification with NaOH. LPCC: Using a column packed with silica gel, elution with n-hexane/acetone (7/3, v/v) and n-hexane/dichloromethane/acetone (5/2.5/1, v/v/v) at constant flow rate of 5 ml/min and 474 nm to monitor the separation process	>96.5% purity, almost exclusively all- <i>trans</i> -N-ASX 3S,3'S isomers	Sun et al. (2015)
<i>H. pluvialis</i>	Purification using activated carbon Column packed with the commercial product darco G-60 at 50, 100, and 200 mg, constant flow of ~1.0 ml/min	N-ASX adsorption capacity was 22.6 mg/g. The purity of the compound was not reported	Casella et al. (2020)
<i>H. pluvialis</i>	Purification by HPCCC The two-phases system was n-heptaneacetonitrile (1:1, v/v), 1,200 rpm, mobile phase at 4 ml/min, 28 °C Additional preparative HPLC: Reverse-phase semipreparative column, 30 °C, mobile phase water-ethanol at 2.5 ml/min in a gradient elution mode	Five purified monoesters (over 98% purities) N-ASX linoleic acid: 80% recovery N-ASX linoleic acid: 80% recovery N-ASX palmitic acid: 73% recovery N-ASX oleic acid: 80% recovery N-ASX stearic acid: 25% recovery	Fábryová et al. (2020)
<i>H. pluvialis</i>	Purification by SAF and two-dimensional liquid chromatography SAF: 30 MPa CO ₂ pressure, 0.05 PLE extract/SC-CO ₂ mass flow rate ratio, 20% (v/v) of water in the feed solution Two-dimensional liquid chromatography Normal-phase: Hypersil gold amino column, 3 μm, elution under isocratic conditions using hexane at 20 μL/min Reverse-phase: Thermo accucore C30 column, 2.6 μm, elution using water/acetonitrile (20:80, v/v) and 2-propanol at 3 ml/min	103.3 mg/g N-ASX Separation of monoesters, diesters and free N-ASX in 25 min	Gallego et al. (2020)
Commercial N-ASX	Purification by semipreparative HPLC Kinetex C18 semipreparative column (5 μm), binary mobile phase 0.1% (v/v) formic acid in water and 95% (v/v) methanol mixed with 5% acetonitrile at 30 ml/min	All- <i>trans</i> N-ASX: 92.9% purity 9- <i>cis</i> N-ASX: 95.3% purity 13- <i>cis</i> N-ASX: 91.8% purity	Yang et al. (2017)
<i>P. rhodozyma</i>	Purification by HSCCC The two-phase system was n-hexane-acetone-ethanol-water (1:1:1:1, v/v/v/v), 850 rpm, mobile phase at 3.0 ml/min, 25 °C Additional silica gel column chromatography	92% 99%	Du et al. (2016)
<i>P. carotinifaciens</i>	Purification by semipreparative HPLC Phenomenex silica gel column (5 μm), 40 °C; mobile phase n-hexane-ethyl acetate-acetone (70:20:10 v:v:v) at 1.2 ml/min	Separation and purification of N-ASX, adonirubin, and adonixanthin isomers	Honda et al. (2020a)

HPCC, high-performance counterchromatography; HSCCC, high-speed countercurrent chromatography; LPCC, low-pressure column chromatography.

phospholipids in an aqueous solution using sufficient energy which can be supplied by ultrasonication, high-pressure homogenization, membrane extrusion, solvent evaporation, electro formation, thin-film dehydration/rehydration, dialysis, and liposome (Mozafari et al., 2008). Ionic gelation is based on the ability to crosslink polyelectrolytes in the presence of multivalent ions such as Ca⁺², Ba⁺², and Al⁺³. In this technique, spherical gel particles are prepared by dripping an aqueous polymer solution into a CaCl₂ solution or preparing an emulsion of the hydrophobic active compound in a polymer solution and dripping it into a calcium solution (Lupo et al., 2014).

According to Adschiri and Yoko (2018), conventional encapsulation techniques have some limitations, such as difficulties in controlling the particle size of the product and the necessity to include some purification steps due to the use of solvents. Supercritical fluids precipitation is a methodology recently reported for the encapsulation of N-ASX. Tirado et al. (2019) used the supercritical emulsions extraction (SEE) technology to encapsulate N-ASX in ethyl cellulose (EC). In this study, the SC-CO₂ was in contact with an emulsion prepared with ethyl acetate (EA), N-ASX, EC (oily phase), and EA-saturated water with tween 80 (water phase). The oily phase solvent and CO₂ were recovered at the top of the

TABLE 6 | N-ASX formulations obtained by different encapsulation techniques.

Formulation	Encapsulation process	Characteristics	References
Nanocapsules of potato protein and N-ASX from <i>H. pluvialis</i>	Coacervation by dropwise addition of N-ASX dissolved in ethanol into potato protein solution and freeze-drying	Potato protein solubilized N-ASX in aqueous solution. Bioaccessibility and bioavailability of encapsulated N-ASX were 11 and 7 fold higher, respectively, than unencapsulated N-ASX	Edelman et al. (2019)
Microcapsules of gelatin, porous starch, and N-ASX from <i>P. rhodozyma</i>	Optimization of gelatin coacervation method. Optimal conditions: Yeast extract 40 ml, gelatin 1.5 g, porous starch 0.5 g, 40 °C, and 2 h	Encapsulation efficiency 88.56%, loading N-ASX 1.55 mg/g, solubility 81.5%. The thermal stability of the molecule increased with the particle size and after 15 days the N-ASX retention was about 90% at 22 and 37 °C	Chen et al. (2019)
Microcapsules of maltodextrin, gelatin, and N-ASX from <i>H. pluvialis</i>	Optimization of microencapsulation by spray drying operated at 180 and 80 °C (inlet and outlet temperature) and flow rate of 300 ml/h. Optimal conditions: Maltodextrine/gelatin ratio 2.1:1 and glycerol monostearate/sucrose fatty acid ether 1.1:1	Encapsulation yield 38.02%, encapsulation efficiency 71.76%, solubility 96.24%, DPPH radical-scavenging activity 96.68%, hydroxyl radical-scavenging activity 86.69%	Zhao et al. (2019)
Nanocapsules of poly (L-lactic acid) (PLLA) and commercial N-ASX	Optimization of supercritical antisolvent conditions to produce the nanocapsules. Optimal conditions: flow rate 1.2 ml/min, temperature 42 °C, pressure 80 bar, volume ratio of acetone/dichloromethane 1:2, and concentration of PLLA 7 mg/ml	Encapsulation efficiency 91.55%, the rates of degradation were 12.4% and 16.5% after 6 months of storage at 0 and 25 °C, respectively	Liu et al. (2019a)
Nanocapsules of ethyl cellulose (EC) and commercial N-ASX	Continuous supercritical emulsion extraction. Conditions: 8 MPa, 311 K, liquid/gas ratio 0.1, CO ₂ flow rate 1.4 kg/h, 0.1%, and oil/water ratio 20:80	Encapsulation efficiency 84%, recovery 90%, EC amount of N-ASX 21 mg/g, released in 10 h 70%, mean size 242 nm	Tirado et al. (2019)
Microcapsules of carboxymethyl cellulose sodium (CMC-Na), microcrystalline cellulose (MCC), and N-ASX from <i>P. rhodozyma</i>	Coacervation with CMC-Na and MCC and freeze-drying. CMC-na/MCC 6:4, acetone/CMC-Na 3:1, CMC-na/water 1:20 and freeze-drying	Encapsulation efficiency 52.88%, solubility 52.88%, N-ASX retention after 55 °C for 16 h 80.86%	Feng et al. (2018)
Nanoscale liposomes of phosphatidylcholine (SPC), cholesterol, and commercial N-ASX	Optimization of the thin-film ultrasound method. Optimal conditions: pH 7.4, ratio of SPC/cholesterol 5:1, ratio of organic phase/water 0.2, and ultrasonic time 4 min	Encapsulation efficiency 97.8%, N-ASX retention rate 82.29% after 15 days at 4 °C, vesicle size 80.62 nm, the encapsulation enhanced the antioxidant activity of the carotenoid	Pan et al. (2018)
Microcapsules of oleoresin, alginate, and N-ASX from <i>H. pluvialis</i>	Optimization of the external ionic gelation method. Optimal conditions: CaCl ₂ concentration 100 mM, oleoresin concentration 50 µg/ml, alginate/oleoresin ratio 3.8, alginate concentration 2.5%, and surfactant concentration 1%	Encapsulation yield 77.2%, release time 56.1 min, particle size 2,200 µm	Niizawa et al. (2019)
Nanocapsules of <i>H. pluvialis</i> N-ASX oleoresin and whey proteins	Emulsification-solvent evaporation technique. Whey protein concentration 1%, oleoresin 4.5%, protein solution/oleoresin ratio 9:1, and ultrasonication for 10 min at 10 W	Encapsulation efficiency 96%, particle size 80–130 nm, bioaccessibility 76%, stability at pH 1–3 and 6–10, N-ASX after 2 h of exposure to UV-B light 70.5%, N-ASX after 24 h in oxidizing conditions 40%	Ha et al. (2019)

column, and nanoparticle suspension was collected at the bottom of the tank. Different emulsions were prepared by varying EC concentrations in the oily phase and surfactant concentration in the water phase, and under the best conditions, the encapsulation efficiency was 84%, and the carotenoid preserved its antioxidant activity. The authors designed a continuous operation that allowed a high production capacity. Liu et al. (2019a) also reported using this technology when preparing microspheres of N-ASX. They optimized the process altering solvent ratio, temperature, pressure, carrier concentration, and flow rate. The N-ASX was dissolved in dichloromethane, acetone, and poly (L-lactic acid) (PLLA), and the solvents were evaporated into the bulk SC-CO₂. The encapsulation efficiency was 91.5%,

and the mean particle size was 954.6 nm. The SC-CO₂ is a new and promising technology in N-ASX encapsulation, and since CO₂ is not used to solubilize the carotenoid, it can be called supercritical antisolvent technology. **Table 6** shows relevant and recent reports of conventional and nonconventional N-ASX encapsulation techniques.

FUTURE PERSPECTIVES AND CONCLUSIONS

Naturally synthesized astaxanthin, N-ASX, has shown superior bioactivity than the synthetically obtained S-ASX. Furthermore, several clinical trials confirm the safety of the natural compound

and its effectiveness for the prevention and treatment of several diseases. Due to this, there is considerable interest in exploiting this compound at the pharmaceutical level. The market value of N-ASX raises to USD15,00.00/kg, while S-ASX costs about USD1,000.00/kg. A comprehensive view, which includes the upstream and the downstream process, must be considered in economic feasibility analysis to establish the N-ASX biotechnological production process. Many efforts need to be made in cell recovery stages, cell disruption, drying, product extraction, purification, and formulation, to make N-ASX production competitive.

Cell harvesting techniques should focus on strategies to avoid the use of centrifugation and filtration. The use of flocculants should be extensively studied in *H. pluvialis* as well as its influence on the molecule purification. On the other hand, the flotation technique has shown very promising results, mostly when used with flocculation. The use of yeasts and bacteria to produce N-ASX may represent an advantage in cell recovery because many yeasts, like *S. cerevisiae*, *Y. lipolytica*, and *K. marxianus* strains, can flocculate. These strains should be selected when generating nonnative N-ASX producing microorganisms. Also, flocculating *E. coli* strains can be generated by overexpressing the native *bcsB* gene (involved in the synthesis of extracellular cellulose) (Ojima et al., 2016).

During the downstream process, the amount of N-ASX obtained is positively related to cell disruption and the extraction method applied since it is an intracellular metabolite. The bead milling and high-pressure homogenization have shown high cell disruption efficiency. However, high equipment investment and high operational costs are the principal disadvantages of these methods. The drying process should not be carried out after cell disruption to reduce production costs and avoid carotenoid degradation. Although SC-CO₂ technology is also expensive, it is being used in the large-scale N-ASX extraction process due to its high extraction efficiency and lack of degradation of the molecule (mild operating conditions). Alternatively, assisted technologies allow cell disruption and N-ASX extraction in a single operation;

they operate under mild conditions, and the processing time can be concise. These technologies have been reported along with enzymes and conventional solvents. However, their use, along with ILs, which are considered “green solvents” and have shown high cell disruption efficiency, is scarcely reported. The bioreactor’s cell culture may be treated immediately by assisted technologies for N-ASX extraction without previous cell disruption and drying. On the other hand, there is a lack of information N-ASX’s extraction from native or nonnative N-ASX-producing bacteria; therefore, specific studies must be carried out in this area.

In pharmaceutical applications, a high-purity product is required. The use of liquid-liquid chromatography combined with liquid-solid chromatography may be useful, since the processes are relatively fast, and 99% purity has been obtained. On the other hand, the stability of the molecule must be guaranteed in pharmaceuticals. Many encapsulation techniques have shown high efficiency, high bioavailability, and stability. The use of SC-CO₂ was recently reported as a high-efficiency encapsulation technique; due to its high cost, this method can be replaced by conventional encapsulation methods, which are efficient and less expensive. The purification and individual formulation of free N-ASX, its esters, or isomers is an opportunity to investigate each molecule’s individual activity and stability.

AUTHOR CONTRIBUTION

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

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

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