Food Chemistry 277 (2019) 128–134

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Comparative assessment on the extraction of carotenoids from microalgal sources: Astaxanthin from *H. pluvialis* and β-carotene from *D. salina*

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ARTICLE INFO

Keywords:
Microalgae
Carotenoids
Haemotococcus pluvialis
Astaxanthin
Dunaliella salina
Beta carotene
Biorefinery

ABSTRACT

Astaxanthin and β-carotene are important carotenoids used in numerous pharmaceutical and nutraceutical applications, owing to their vigorous antioxidant properties. The microalgal strains *Haematococcus pluvialis* and *Dunaliella salina* accumulate the highest quantities of astaxanthin and β-carotene (up to 7% and 13% dry weight respectively) and are therefore considered as sustainable feedstock for the commercial production of carotenoids. Thus, from an economical perspective, it becomes desirable to optimize recovery of carotenoids from microalgal cells. To this end, here, we have summarized the conventional and modern extraction techniques generally used for the recovery of astaxanthin from *Haematococcus pluvialis* and β-carotene from *Dunaliella salina*. Furthermore, we have also discussed the optimum process conditions employed for numerous extraction protocols including solvent extraction, ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE). Overall, our study highlights the sustainability of integrated co-production of biofuels and carotenoids in a biorefinery framework.

1. Introduction

Microalgae are a diverse group of unicellular species with a microscopic structure which allows them to convert sunlight into chemical energy with the use of a carbon source. There are more than 30,000 of known species of microalgae growing in freshwater and marine environments (Shah, Liang, Cheng, & Daroch, 2016). The distribution of microalgae in different ecosystems leads to diverse chemical compositions, making them attractive for bioprocessing.

Being photosynthetic unicellular species, microalgae have inherited a balanced combination of both micro-organic and higher plant properties (Milledge, 2010; Satyanarayana, Mariano, & Vargas, 2011). Similar to other microorganisms, microalgae exhibit fast growth rates and synthesize secondary metabolites. Furthermore, microalgae grow with simple nutritional requirements and performs efficient oxygenic photosynthesis similar to higher plants. Besides, microalgae are commercially advantageous to cultivate for industrial applications due to their capability to use CO_2 as the only carbon source, light as the solitary energy source and no requirement of arable lands (Bux & Chisti, 2016; Pragya, Pandey, & Sahoo, 2013).

These attributes make microalgae eligible candidates for the commercial production of high-value compounds including carotenoids, bioactive peptides and polyunsaturated fatty acids (PUFA) (Borowitzka, 2013; Christaki, Florou-Paneri, & Bonos, 2011). Among them, carotenoids have surpassed the value of other microalgal chemical recoveries due to their intrinsic antioxidant, antitumoral and anti-inflammatory features which have numerous applications in pharmaceutical, food and cosmetic industries (Irwandi, 2011; Spolaore, Joannis-Cassan, Duran, & Isambert, 2006).

2. Carotenoids: natural synthesis in microalgae

Carotenoids are one of the three prominent groups of natural pigments present in microalgae other than the chlorophylls and phycobiliproteins (Guedes, Amaro, & Malcata, 2011). In general, carotenoids absorb light in wavelengths 400–550 nm; a range which chlorophyll pigments are incapable of utilizing for photosynthetic metabolism (Gong & Bassi, 2016). Primary carotenoids such as β-carotene, lutein and violaxanthin reduce the excess energy requirement in photosynthesis by transferring absorbed energy to chlorophylls (Raposo, de Morais, & de Morais, 2015). Hence, primary carotenoids serve as complementary pigments which expand the light absorbing spectrum of microalgae. Secondary carotenoids such as astaxanthin and canthaxanthin protect chlorophyll from photodamage by forming a protective layer over microalgal cells when they are exposed to extreme light intensities (Gong & Bassi, 2016). Thus, under stressed culture conditions,

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https://doi.org/10.1016/j.foodchem.2018.10.066
Received 19 May 2018; Received in revised form 10 October 2018; Accepted 12 October 2018
Available online 13 October 2018

secondary carotenoids are synthesized in higher quantities, performing a protective mechanism for microalgal cells.

Moreover, carotenoid pigments demonstrate antioxidant properties to protect microalgae from free radical attacks and stabilize cellular metabolic functions. For instance, carotenoids are capable of preventing oxidative degradation of intracellular lipids in microalgae by scavenging reactive radicals using their intrinsic functional groups (Bhosale & Bernstein, 2005; Varela, Pereira, Vila, & León, 2015).

2.1. Chemistry of carotenoids

The general structure of carotenoids contains a molecular backbone derived from a forty carbon polyene chain with conjugated double bonds and terminal carbon rings. The distinctive properties of each type of carotenoid are derived from the cyclic hydrocarbon groups and oxygen containing functional groups attached to its molecular backbone (Chandi & Gill, 2011; Riccioni, D'Orazio, Franceschelli, & Speranza, 2011). According to the constituent functional groups and the basic chemical structure, carotenoids can be classified as carotenes and xanthophylls. Carotenes such as β-carotene ($C_{40}H_{56}$) are purely hydrocarbon carotenoids whereas xanthophylls such as astaxanthin ($C_{40}H_{52}O_4$) comprise of oxygenated derivatives including hydroxyl (–OH) and ketone (–CO) substituents (Gateau, Solymosi, Marchand, & Schoefs, 2016). Table 1 depicts the chemical structures of carotenoids representing carotenes and xanthophylls.

3. Carotenoids from different microalgal sources

Generally, the predominant carotenoid amounts to approximately 70% of the total carotenoid content or 3–5% of dry weight in microalgae while the residual fraction is made up of minor quantities of other carotenoids (Table 2) (Guedes, et al., 2011). For instance, 45% of total carotenoids in *Chlorella vulgaris* is canthaxanthin while astaxanthin and violaxanthin make up the minor fractions. On the contrary, β-carotene in *Dunaliella salina* and astaxanthin in *Haematococcus pluvialis* have been reported to exceed 80% of the total carotenoids present (Raposo et al., 2015). Therefore, these strains have been the subject of extensive research focusing on improving the extractability of astaxanthin and β-carotene.

Besides, astaxanthin and β-carotene have acquired a strong and escalating market demand to date. According to forecasts made by Zion Market Research Global in 2016 (Joel, 2016), the market value of carotenoids is anticipated to evolve at a compound annual growth rate of 3.5% from 2016 to 2021 while reaching a revenue of USD 1.52 billion in 2021. According to Fig. 1, β-carotene and astaxanthin are expected to achieve market shares of 26% and 25% by 2021 (Joel,

Table 2
Carotenoids synthesized by different types of microalgae (Raposo & Morais, 2015).

Microalgae species	Carotenoids	
	Main product	By-products
Dunaliella salina	β-carotene	zeaxanthin, lutein, α-carotene
Haematococcus pluvialis	astaxanthin	β-carotene, cantaxanthin, lutein
Chlorella vulgaris	canthaxanthin	astaxanthin, violaxanthin
Scenedesmus almeriensis	lutein	β-carotene
Corymbia ellipsoidea	violaxanthin	antheraxanthin, zeaxanthin

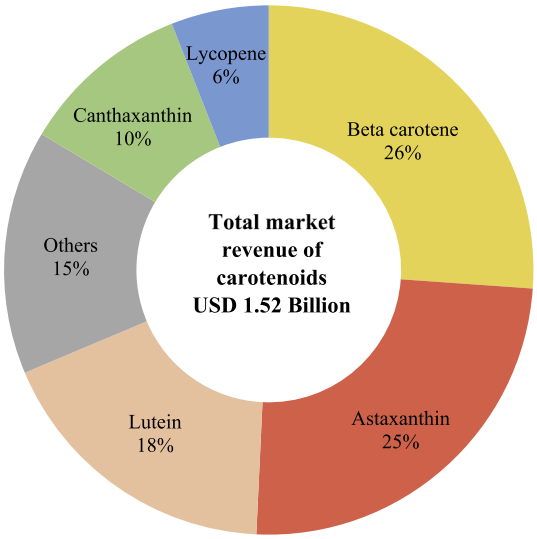
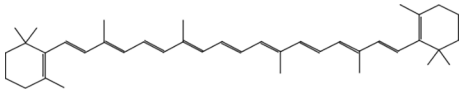
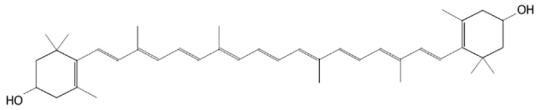
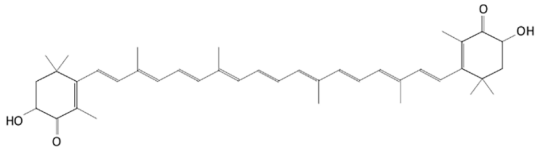
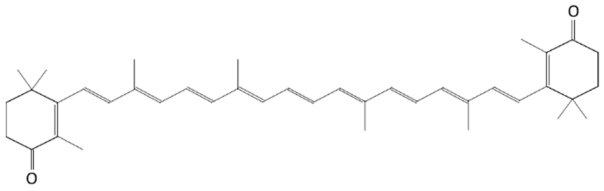


Fig. 1. Prospective global carotenoids market revenue in 2021, with respect to the carotenoid type as described by Zion Market Research Global in September 2016 (Joel, 2016).

Table 1
Chemical structures of carotenoids along with the IUPAC names (Raposo & Morais, 2015).

Carotenoid	Chemical structure	IUPAC name
β-Carotene		β, β-carotene
Lutein		β, ε-carotene-3,3′-diol
Astaxanthin		3,3′-dihydroxy-β, β-carotene-4,4′-dione
Canthaxanthin		β, β-carotene-4,4′-dione

2016). Due to the high accumulation of carotenoids and projected market demand, *Haematococcus pluvialis* and *Dunaliella salina* are considered as promising microalgal sources for industrial bioprocessing.

3.1. Astaxanthin ($C_{40}H_{52}O_4$)

Oxidative stress; the disturbance in balance between the occurrence of free radicals and the capacity of the body to detoxify them, leads to several non-infectious diseases including cancer, chronic inflammation and cardiovascular diseases (Milledge, 2010). Astaxanthin is considered as the most powerful antioxidant in nature, serving the role of a highly efficient scavenger of free radicals that build up within the human body. The presence of hydroxyl (–OH) and ketone (–CO) functional groups, makes the structure of astaxanthin polar and susceptible to oxidation, thereby imparting antioxidant properties (Dufossé et al., 2005; Hussein, Sankawa, Goto, Matsumoto, & Watanabe, 2006). Therefore, astaxanthin is used as a human dietary supplement and potent quenchers of singlet oxygen in medical treatment processes. However, unlike other known carotenoids, astaxanthin does not serve as a vitamin A precursor (Murthy, Rajesha, Swamy, & Ravishankar, 2005; Raposo et al., 2015).

Astaxanthin is synthetically produced using petrochemical sources which have issues of food safety, potential toxicity in the final product, pollution and sustainability (Hamed, 2016). Therefore, synthetic astaxanthin has not been approved by the Food and Drug Administration (FDA) in USA for direct human consumption in food or dietary supplements to date (Guedes, et al., 2011). Therefore, commercial production of natural astaxanthin is now acquiring emphasis over synthetic production.

In this scenario, microalgae play a vital role with their potential to synthesize astaxanthin as a secondary metabolite. *Chlorella vulgaris, Chlorella zofingiensis, Chlorococcum wimmeri*, *Botryococcus braunii* and *Scenedesmus obliquus* are some of the notable microalgal strains that have the potential to produce astaxanthin (Markou & Nerantzis, 2013). However, *Haematococcus pluvialis* cells are capable of accumulating a comparatively higher amount of astaxanthin; up to 7% of dry biomass and 90% of its total carotenoids (Pulz & Gross, 2004; Raposo et al., 2015). Thus, *Haematococcus pluvialis* is the most favorable microalgal species for industrial scale production of natural astaxanthin.

Being a secondary metabolite, synthesis of astaxanthin in *Haematococcus pluvialis* is induced by extreme growth conditions such as nitrogen and phosphorus starvation, high solar intensities, salt stress and elevated temperature (Shah, et al., 2016; Solovchenko, 2015). Astaxanthin primarily accumulates in the cytoplasm of *Haematococcus pluvialis* cells as a racemic mixture of mono- and di-esters (up to 97% of the total astaxanthin production), while a minute fraction of the yield is present as free astaxanthin. Furthermore, β-carotene, lutein, canthaxanthin and neoxanthin are recovered as minor derivatives in the process of astaxanthin extraction (Gong & Bassi, 2016).

3.2. β-Carotene ($C_{40}H_{56}$)

β-Carotene is the first ever high-value product to be commercially produced from microalgae (Gateau, et al., 2016). Besides being an antioxidant, it serves as a precursor for vitamin A biosynthesis within the human body (Hu, Lin, Lu, Chou, & Yang, 2008). Natural β-carotene prevents the decline of white blood cells and platelets caused by ionic radiation, thus boosting the immunity system and providing protection against undesirable radiation exposures (Irwandi, 2011; Vílchez et al., 2011). Medical treatments associated with β-carotene have shown its ability to successfully inhibit and prevent various types of tumors in the human body. Moreover, β-carotene is effective in controlling cholesterol levels and reducing the risk of cardiovascular diseases (Gateau, et al. 2016; Pangestuti & Kim, 2011).

Chlorella zofingiensis, Spirulina platensis and *Caulerpa taxifolia* synthesize β-carotene at an average yield of 0.1–2% of its dry biomass weight. However, the marine microalgae species *Dunaliella salina* has a β-carotene yield up to 13% of its dry biomass and is consequently used as the predominant source for commercial production of natural β-carotene (Raposo et al., 2015).

The analysis of total carotenes of *Dunaliella salina* has revealed that the typical composition of the β-carotene synthesis is approximately 42% of all-*trans* β-carotene, 41% of 9-*cis* β-carotene, 10% of 15-*cis* β-carotene and 6% of other isomers (Ben-Amotz, 1980; Rigo Roso, 2015). In contrast, the synthetic production of β-carotene is limited to its all-*trans* isomer (Ye, Jiang, & Wu, 2008). Therefore, the natural production of β-carotene has the advantage of being the unique source of 9-cis β-carotene. The natural 9-cis isomer of β-carotene plays a major role in quenching oxygen free radicals and preventing oxidative damage to the cell. Hence, natural β-carotene is considered superior to synthetic products and holds the primacy of higher market demand (Ye, et al., 2008).

β-Carotene is accumulated as droplets in the chloroplast stroma of *Dunaliella salina* cells, particularly when exposed to extreme culture conditions such as high temperatures, high light intensities, high levels of salinity and deficiency of nitrogen sources (Kleinegris, Janssen, Brandenburg, & Wijffels, 2011; Wichuk, Brynjólfsson, & Fu, 2014). Furthermore, *Dunaliella salina* is capable of growing in high salt concentrations where few other organisms can survive. Therefore, the industrial cultivation of *Dunaliella salina* in excessive salt concentrations results in high yield of β-carotene while reducing the risk of culture contamination by other aquatic organisms.

4. Extraction of carotenoids from microalgae

Due to the vast distribution of microalgal carotenoids with various chemical properties, it is impractical to develop a general extraction protocol. Thus, for determining the most appropriate extraction technique, it is crucial to assess the chemical structure of the carotenoid and the nature of the host microalgal species. The foremost methods to recover carotenoids from microalgae are solvent extraction and the supercritical fluid extraction (SFE).

4.1. Solvent extraction

Solvent extraction is a relatively simple and economical technique conventionally used to extract carotenoids. However, the process may become disadvantageous if the organic solvents are expensive, hazardous or used in large quantities. Solvent extraction typically requires multiple extraction steps to achieve the desired level of carotenoid recovery, making it a time-consuming method. Pretreatment is employed for disruption of the cell membrane, improving the mobility of carotenoids into the extraction solvent, thereby enhancing extraction efficiency. Pretreatment consists of mechanical methods including grinding, bead milling, ultrasonication and high-pressure homogenizing; and non-mechanical methods including enzymatic or chemical hydrolysis and osmotic shocks (Desai, Streefland, Wijffels, & Eppink, 2016).

Even though it is impractical to develop a common extraction method for recovery of natural carotenoids, Kagan and Braun (2001) have proposed a general method using a multi-phase solvent mixture. The carotenoid source is treated at an elevated temperature using a multi-phase solvent mixture composed of water, a hydrophobic solvent and an aqueous co-solvent. Methyl acetate, edible oils and chloroform are the most common hydrophobic reagents used in the multi-phase solvent mixture, selected based on molecular polarity and extraction efficiency. In contrast, the aqueous co-solvent is selected based on the limited extractability of carotenoids. Thus, the aqueous co-solvent should be an alcohol, ether or ketone (i.e. methanol, *n*-propanol, ethylene glycol etc.) (Kagan & Braun, 2001). The extraction process is conducted at temperatures exceeding 50 °C, preferably 65 °C, for one hour to extract carotenoids from microalgae into the solvent mixture

which is agitated throughout the entire treatment period (Kagan & Braun, 2001).

4.2. Supercritical fluid extraction (SFE)

SFE technology enables the rapid recovery of carotenoids with a higher efficiency than conventional solvent extraction. The most common fluid used as the extraction solvent is supercritical CO_2; which can selectively recover carotenoids by controlling solvent density (Herrero, Cifuentes, & Ibanez, 2006). The time required for extraction is lowered as the higher diffusion coefficient and lower viscosity of supercritical CO_2 allows rapid penetration into the pores of extracellular matrices (Saini & Keum, 2018). Furthermore, SFE is considered a green process due to the potential of CO_2 recycling and elimination of hazardous organic solvents (Machmudah, Shotipruk, Goto, Sasaki, & Hirose, 2006).

However, SFE tends to recover chlorophylls more efficiently than carotenoids which may lead to the production of extracts with relatively poor purity (Herrero, et al., 2006). Moreover, SFE has high capital and operating costs due to the high pressures required for maintenance of extraction solvents at their supercritical state. Thus, widespread adoption of SFE as a substitute for conventional solvent extraction is held back by economic considerations.

4.3. Astaxanthin from Haematococcus pluvialis

Due to the lipophilic property of astaxanthin, solvent extraction is the principal method utilized for its recovery from *Haematococcus pluvialis*. In addition, a post-hydrolysis of the extracted astaxanthin is necessary due to its esterified accumulation in *Haematococcus pluvialis* cells (Gong & Bassi, 2016).

When encysted *Haematococcus pluvialis* cells are treated with 40% (v/v) acetone at 80 °C for 2 min followed by lyophilization or cell treatment with lytic enzymes, 70% of astaxanthin can be recovered (Kobayashi, Kurimura, Sakamoto, & Tsuji, 1997). The high temperature treatment process enables the removal of chlorophyll pigments from cyst cells, thus increasing the purity of the extract.

Alternatively, ionic liquids such as 1-ethyl-3-methylimidazolium dibutylphosphate (EMIM DBP) can be utilized to improve the permeability of encysted *Haematococcus pluvialis* cells at mild temperatures. Desai, et al. (2016) have observed that the treatment of dried microalgal cells with aqueous EMIM DBP at 40% (w/w) and 45 °C for 90 min followed by solvent extraction with ethyl acetate resulted in an enhanced yield of astaxanthin, in excess of 70% (Desai, et al., 2016).

Furthermore, a two-stage solvent extraction process employing dodecane and methanol permits the recovery of astaxanthin from *Haematococcus pluvialis;* excluding the cell harvesting step. In the first stage, the culture broth is treated with dodecane and gravity settling is used to separate the extract from the culture medium. In the second stage, the dodecane extract is treated with 0.02 M NaOH in methanol at 4 °C to saponify the astaxanthin esters and selectively extract free astaxanthin to the methanol phase. Thus, due to mild conditions employed in the two-stage solvent extraction process, degradation of free astaxanthin is minimized with a total yield of 85% (Kang & Sim, 2007).

The acidic extraction method by Sarada, Vidhyavathi, Usha, and Ravishankar (2006) has been successful in improving the extractability of astaxanthin up to 86–94% from *Haematococcus pluvialis*. First, the algal biomass has been lyophilized and treated with HCl (4 M, 1 ml per 10 mg of biomass) at 70 °C for 2 min followed by centrifugation at 5000 rpm for 10 min. Thereafter, the solution has been ultrasonically treated at 0 °C with acetone followed by centrifugation at 5000 rpm and 4 °C for 6 min. Furthermore, it has been observed that the acidic treatment has no effect on the ester profile of natural astaxanthin (Sarada, et al., 2006).

Moreover, Dong, Huang, Zhang, Wang, and Liu (2017) have employed a binary solvent mixture of hexane and isopropanol in the volume ratio of 6: 4 (v/v) to extract astaxanthin from lyophilized microalgal biomass with ultrasonic assistance for 20 min. Cell biomass and the extract have been separated by centrifugation at 3500 rpm and 4 °C for 5 min, followed by vacuum concentration (Dong, et al., 2017).

Microwave-assisted extraction is considered an efficient and economic method for the recovery of astaxanthin from *Haematococcus pluvialis*. The transmission of microwave energy through microalgal biomass increases the intracellular kinetic energy and causes vibration of liquid particles. The subsequent increase in cellular temperature and exertion of pressure on the cell walls lead to the disruption of cells. Furthermore, the microwave-assisted process reduces extraction time by breaking the intermolecular bonds of the extraction solvent and initiating the transfer of dissolved ions through the pores of the cellular matrices (Saini & Keum, 2018). Liyan, Guitang, Guanghua, and Xiaosong (2009) have deduced that employing optimized extraction conditions; treatment with ethyl acetate at 141 W microwave power for 83 s results in a recovery of 594 μg of astaxanthin per 100 mg of *Haematococcus pluvialis* biomass (Liyan et al., 2009).

Ultrasound-assisted extraction is a modern technique which facilitates efficient recovery of astaxanthin from *Haematococcus pluvialis*. The impact of ultrasound on astaxanthin extraction is attributed to acoustic cavitation which causes rapid cell wall disruptions, thus enhancing the mass transfer of extractants into the cell matrices (Saini & Keum, 2018). Ultrasonic power, temperature and solvent density are considered as critical parameters in the extraction of astaxanthin. An astaxanthin yield of 27.58 mg per 1 g of dried *Haemotococcus pluvialis* biomass has been achieved by Zou, Jia, Li, Wang, and Wu (2018) using ultrasound-assisted extraction with 48% ethanol in ethyl acetate, liquid-to-solid ratio of 20:1 (mL/g), extraction time of 16.0 min at 41.1 °C and ultrasound power of 200 W (Zou et al., 2018).

Pour, Tavakoli, and Sarrafzadeh (2017) have observed that employing SFE parameters of 400 bar and 40 °C under a CO_2 flowrate of 3 $cm^3 min^{-1}$ with 1.67% (v/v) ethanol resulted in 80.6% extractability of astaxanthin from *Haematococcus pluvialis* (Pour, et al., 2017). The quantity of astaxanthin in the extract can be increased by using high CO_2 flowrates and elevated temperatures. However, upon reaching the saturated state of the supercritical fluid, the astaxanthin content of the extract cannot be increased further (Herrero, et al., 2006).

Table 3(A) depicts a comparison of methods used for extraction of astaxanthin from *Haematococcus pluvialis*.

4.4. β-Carotene from Dunaliella salina

Similar to the extraction of astaxanthin from *Haematococcus pluvialis,* majority of protocols used for the recovery of β-carotene from *Dunaliella salina* involve solvent extraction due to its low cost and simplicity. However, the presence of chlorophyll in *Dunaliella salina* interferes with the direct extraction of β-carotene. The treatment of microalgal biomass with calcium hydroxide facilitates the conversion of chlorophyll into calcium salts which are insoluble in the solvents utilized for the extraction of β-carotene (Hoffmann, 1984). Hence, it becomes feasible to extract β-carotene from *Dunaliella salina* without chlorophyll contamination.

A patented method developed by Rudolf Ruegg discloses an extraction method for β-carotene from algal sources. Chlorophyll in microalgae is saponified by treatment with calcium hydroxide at 50 °C–100 °C under an inert gas atmosphere for 2–6 h (Hoffmann, 1984). Thereafter, β-carotene is extracted from the filtered residue of the saponification reaction using a halogenated hydrocarbon solvent such as methylene chloride or a hydrophobic solvent such as hexane or petroleum ether. Moreover, glycerine can be co-produced from the filtrate of saponification by pH neutralization followed by extraction with a lower alkanol solution (Hoffmann, 1984).

The pressured fluid extraction method employed by Abu-Rezq, Al-Hooti, Jacob, and Ahmed (2010) facilitates the extraction of β-carotene from *Dunaliella salina* by the filtration of microalgal biomass suspended

Table 3
Comparison of extraction methods of astaxanthin from *Haematococcus pluvialis* and β-Carotene from *Dunaliella salina*.

Extraction process	Process conditions	Steps for recovery enhancement	Product recovery	Remarks	Ref
(A) Astaxanthin from *Haematococcus pluvialis*	Treatment with 40% (v/v) acetone at 80 °C for 2 min	Cell treatment with lytic enzymes or lyophilization	70%	Possible to remove chlorophyll pigments from biomass	Kobayashi, et al. (1997)
	Heating with 40 percent (w/w) EMIM DBP at 45 °C for 90 min	Centrifugation followed by treatment with ethyl acetate	More than 70%	Involvement of mild temperature with high recovery	Desai, et al. (2016)
	Treatment of culture broth with dodecane followed by methanol	Saponification of astaxanthin-esters by the addition of 0.02 M NaOH at 4 °C	85%	Extraction is possible without cell harvesting with minimum degradation of free astaxanthin	Kang and Sim (2007)
	Lyophilization followed by treatment with 4 M HCl at 70 °C for 2 min	Solvent treatment with acetone at 0 °C followed by centrifugation at 4 °C for 6 min	86 to 94%	No effect on the ester profile, hence advantageous in preservation	Sarada, et al. (2006)
	Lyophilization followed by treatment with hexane and isopropanol 6:4 (v/v)	Ultrasonic treatment at 0 °C followed by centrifugation at 3500 rpm for 5 min	9.7 mg per g of dry biomass	A binary organic solvent extraction method which allows the removal of chlorophyll	Dong, et al. (2017)
	Microwave assisted extraction with ethyl acetate	Treatment with 98% ethyl acetate at 141 W for 83 s	594 μg per 100 mg of dry biomass	High extraction efficiency with low consumption of organic solvents	Liyan et al. (2009)
	Ultrasound assisted extraction with ethanol in ethyl acetate	Treatment with 48% ethanol in ethyl acetate at 200 W and 41.1 °C for 16 min	27.58 mg per g of dry biomass	High extraction efficiency with low degradation	Zou et al. (2018)
	Supercritical CO_2 fluid extraction at a flowrate of $3cm^3min^{-1}$	Cell treatment at 400 bar and 40 °C with 1.67% (v/v) ethanol	80.6%	Highly efficient and environmentally friendly extraction process	Machmudah, et al. (2006)
(B) β-carotene from *Dunaliella salina*	Treatment with pure acetone	Filtration using a 0.45 μm membrane	33.8–96.5 pg per cell	Higher recoveries through pressured fluid extraction method	Abu-Rezq, et al. (2010)
	Heating with $Ca(OH)_2$ at 50 °C to 100 °C for 2 to 6 h	Filtration followed by the treatment with a halogenated hydrocarbon	Not mentioned	Possible to obtain glycerin as a byproduct from the filtrate	Kagan and Braun (2001)
	Supercritical CO_2 fluid extraction at a flowrate of $3cm^3min^{-1}$	Cell treatment at 400 bar and 55 °C with 100% supercritical CO_2	115.43 μg per g of dry biomass	Green extraction process with high recovery	Pour, et al. (2017)
(C) General extraction of carotenoids	Stirring with a multi-phase solvent mixture at 65 °C for one-hour duration	Treatment with a hydrophobic carotenoid solvent and an aqueous co-solvent	Not mentioned	General method applicable for carotenoid extraction from natural sources	Kagan and Braun (2001)

in pure acetone through a 0.45 μm membrane. It has been observed that the recovery of β-carotene was in the range of 33.8–96.5 pg per microalgal cell (Abu-Rezq, et al., 2010).

Alternatively, β-carotene can be extracted from *Dunaliella salina* by agitation of microalgae biomass at 30 °C in a solvent mixture of acetic esters of C_1–C_4 alcohols and 25% (w/w) natural oils such as sunflower seed oil. The novelty of this method is the combination of two water immiscible solvents in the carotenoid extraction process (Jurgen, Huber, Kohlrausch, & Jan, 1998). In contrast, the method patented by Kagan and Braun describes the extraction of β-carotene from halophilic algae including *Dunaliella salina* by membrane ultrafiltration of an aqueous suspension of biomass emulsified with edible oil at elevated temperatures (Kagan & Braun, 2001).

Moreover, in a study by Pour, et al. (2017), SFE parameters of 400 bar and 55 °C has been used to yield 115.43 μg of β-carotene per g of *Dunaliella salina* dry biomass. When the effect of supercritical fluid parameters is considered, pressure has been more significant on the yield of β-carotene than temperature (Pour, et al., 2017). Hence, the operating conditions of the extraction process must be stabilized at their optimum values for the maximum recovery of β-carotene from *Dunaliella salina*.

Table 3(B) compares extraction protocols employed for recovery of *β*-carotene from *Dunaliella salina*.

5. Production of carotenoids in a biorefinery framework

In contrast to terrestrial plants, microalgae do not necessarily require freshwater or arable lands for growth (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011; Shah, et al., 2016). Furthermore, biomass can be cultivated in waste streams containing sufficient nutrients and a suitable carbon source such as flue gas from power generation. Hence, microalgae can be integrated for sequestration of carbon dioxide emissions and wastewater treatment co-currently with generation of biomass (Mata, Martins, & Caetano, 2010).

In spite of these advantages, the costs incurred in cultivation and subsequent downstream processes accounts for 50–90% of the total expenditure of manufacturing microalgae based bioproducts depending on the purity and biochemical properties required (Harun, Singh, Forde, & Danquah, 2010). Although microalgal biofuel has great potential, widespread production is hindered by economic infeasibility. However, this can be mitigated by the extraction of lucrative high value products such as carotenoids, PUFA and bioactive peptides from microalgal biomass used to manufacture biofuels. Therefore, the integrated co-production of high-value compounds with biofuels in microalgae based biorefineries (Fig. 2) can be considered as a potential solution towards sustainability.

The high accumulation of astaxanthin in *Haematococcus pluvialis* and β-carotene in *Dunaliella salina* makes them viable microalgal strains for commercial carotenoid production (Guedes, et al., 2011). The global market value for astaxanthin was estimated at USD 555.4 million in 2016 while β-carotene was valued at USD 432.2 million in 2015 (Grand view research inc, 2016; Joel, 2016). Hence, with high productivity and high market value for astaxanthin and β-carotene, industrial scale biorefineries of *Haematococcus pluvialis* and *Dunaliella salina* are potentially remunerative despite expensive cultivation systems and downstream processes.

The growth of *Haematococcus pluvialis* and *Dunaliella salina* under nutrient starvation promotes the synthesis of carotenoids as well as the accumulation of triglycerides (Minhas, Hodgson, Barrow, & Adholeya, 2016). Therefore, by employing nutrient stressed conditions, carotenoid-rich biomass of *Haematococcus pluvialis* and *Dunaliella salina* can be synthesized with significant fatty acid contents up to 30–60% of dry weight (Shah, et al., 2016; Ye, et al., 2008). Hence, lipids extracted from residual biomass, following carotenoid recovery, can be employed for co-production of biodiesel through transesterification. Furthermore, gasification of residual biomass from lipid extraction can yield synthesis

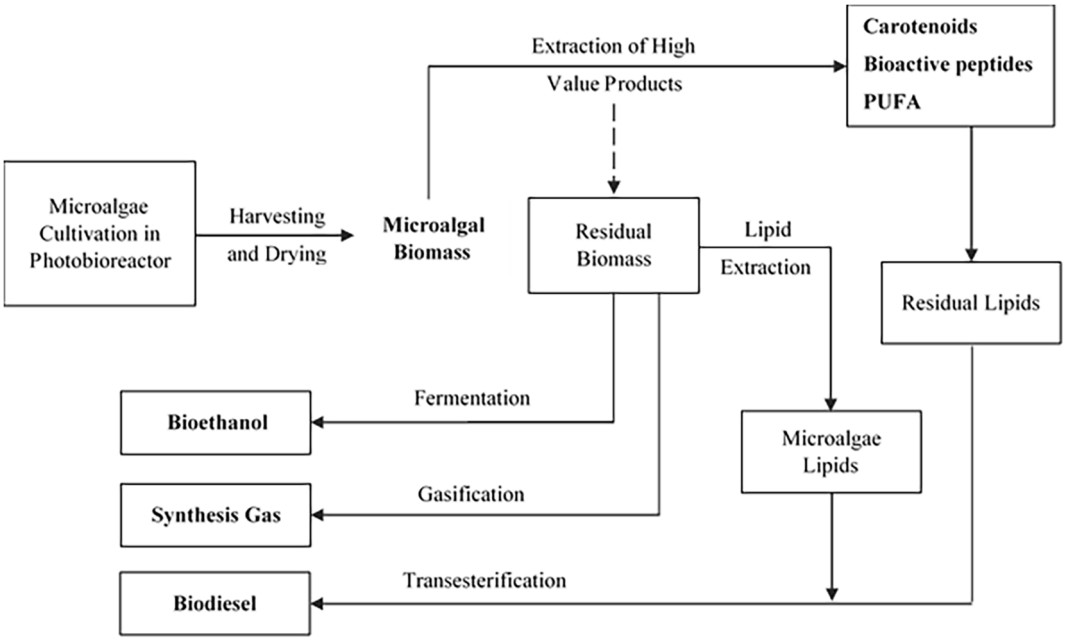


Fig. 2. Typical approach for a microalgae-based biorefinery system.

gas. Energy can be recycled into the biorefinery process by combustion of syn gas thus produced, improving the cost-effectiveness of the overall process. Alternatively, depending on microalgal composition, bioethanol can be produced by fermentation of the carbohydrate fraction of residual biomass from carotenoid recovery (Yen et al., 2013). The carbohydrate content in *Haematococcus pluvialis* and *Dunaliella salina* can reach up to 50–60% of dry weight, thereby making bioethanol a viable by-product in biorefineries (Doan, Moheiman, Mastrangelo, & Lewis, 2012).

Therefore, *Haematococcus pluvialis* and *Dunaliella salina* are two microalgal strains which can synthesize promising feedstock for the development of biorefineries with astaxanthin and β-carotene as main products. However, further studies must be conducted to assess the economic feasibility and environmental impact of such biorefineries.

6. Conclusions

Conventionally, the carotenoid demand for pharmaceutical and nutraceutical sectors is fulfilled from fatty fish. However, excessive harvesting of sea fish for their health benefits is an unsustainable practice due to the interruption of ecological balance. Hence, it is important to consider alternative sources such as microalgae to produce carotenoids. Astaxanthin and β-carotene have the highest market demand in the global carotenoid market, with *Haematococcus pluvialis* and *Dunaliella salina* being the two strains exhibiting the highest natural accumulation of respective carotenoids.

Astaxanthin and β-carotene are conventionally extracted using organic solvents due to their hydrophobic nature. The extraction solvent is selected based on chemical properties of the target carotenoid. Solvent extraction is widely adopted in commercial production of carotenoids due to its relative simplicity and low energy requirement. Alternatively, supercritical fluid extraction (SFE) is a rapid, environmentally friendly process that is gaining traction but is held back by concerns of product purity and cost implications.

Residual biomass of *Haemotococcus pluvialis* and *Dunaliella salina* from carotenoid extraction is viable for production of biodiesel due to the high content of fatty acids. Therefore, the impact of high costs associated with downstream processes can be mitigated by co-production of carotenoids and biofuels in a biorefinery framework. Hence, mass scale implementation of microalgal biorefinery systems can lead to a highly lucrative production process compared to the use of biomass for the manufacture of a solitary product.

Nonetheless, further technological advancements are required for the extraction of astaxanthin from *Haemotococcus pluvialis* and β-carotene from *Dunaliella salina* to increase extraction efficiency, ensure food safety and develop potential biorefinery routes.

Acknowledgement

This review was supported by the Ministry of Science, Technology and Research, Sri Lanka under the framework of the project “Process development and assessment/evaluation of microalgae based biofuels and value-added products” (Grant No. MSTR/TRD/AGR/3/2/18).

Declarations of interest

The authors have declared that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.10.066.

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