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Review Article

Sustainable production of food grade omega-3 oil using aquatic protists: Reliability and future horizons

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ABSTRACT

Biotechnological production of omega-3 polyunsaturated fatty acids (PUFAs) has become a commercial alternative to fish oil in the past twenty years. Compared to PUFA production by fatty fishes, that from microorganisms has increased due to its promising sustainability and high product safety and to increasing awareness in the expanding vegan market. Although autotrophic production by microalgae seems to be more sustainable in the long term, to date most of the microbial production of omega-3 is carried out under heterotrophic conditions using conventional fermentation technologies. The present review critically analyzes the main reasons for this discrepancy and reports on the recent advances and the most promising approaches for its future development in the context of sustainability and circular economy.

Introduction

Omega-3 polyunsaturated fatty acids are recognised as fundamental elements in the human diet, with a series of health effects and benefits in the treatment of several pathologies. The low ratio between omega-6 and omega-3 series in the modern diet involves an increase in the risks of cardiovascular diseases, type 2 diabetes and some types of cancer in genetically predisposed individuals [1]. Alpha-linolenic acid (ALA, C18:3 n-3) is an omega-3 PUFA found in some biomass such as walnuts, flax, soybean, chia, hemp and *Echium plantagineum* L. [2]. ALA is the precursor for the biosynthesis of omega-3 very long chain polyunsaturated fatty acids (VLC-PUFAs) such as stearidonic acid (SDA, C18:4), eicosapentaenoic acid (EPA, C20:5), docosapentaenoic acid (DPA, C22:5) and docosahexaenoic acid (DHA, C22:6).

For most animals, VLC-PUFAs are essential components of cell membranes in neural and muscle tissues and are precursors of signalling molecules (bioactive lipid mediators) [3]. Moreover, DPA is the second most frequent constituent of the human brain and is important in pregnancy and foetal neural development [4]. Nevertheless, *de novo* synthesis of EPA and DHA is only performed efficiently by some taxa of aquatic protists, generally described as microalgae, representing the main source of these fatty acids in the biosphere [5]. VLC-PUFAs are transferred through trophic chains to invertebrates and fish, and then to terrestrial consumers, including humans. Terrestrial plants do not produce VLC-PUFAs and most vertebrates, including humans, cannot synthesise the conversion of ALA to DHA efficiently due to the lack or poor expression of the required enzymes [5]. Thus, EPA, DPA and DHA are considered essential fatty acids because they must be obtained from food or supplementary sources [1].

Although humans are genetically adapted to a ratio of omega-6/omega-3 fatty acids of about 4 to 1, to date the worldwide availability of VLC-PUFAs seems insufficient to meet the demand [6]. The main source of VLC-PUFAs in the human diet is fish oil. Aquaculture of fatty fish rich in omega-3 depends on the fish forage that provides fish meal and fish oil, the key fish feed ingredients. Coupled with fisheries for direct human consumption, this affects the fish stocks that are predicted to be irreversibly damaged in the near future [7]. Therefore, in order to fight the rising cost of fish oil, the content of vegetable biomass in fish diets is progressively increasing, resulting in a lower VLC-PUFA content in fish muscle [8].

Abbreviations: AD, anaerobic digestion; ALA, alpha-linolenic acid; APC, allophycocyanin; CSL, corn steep liquor; CW, cheese whey; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EM, Embden-Meyerhof; EPA, eicosapentaenoic acid; EPS, extracellular polymeric substances; FAN, free amino nitrogen; FBW, food by-products and waste; FX, fucoxanthin; MPBR, membrane photobioreactor; PBP, phycobiliprotein; PBR, photobioreactor; PE, phycoerythrin; PKS, polyketide synthase; PP, pentose phosphate; PUFA, polyunsaturated fatty acid; VLC-PUFA, omega-3 long chain polyunsaturated fatty acid; TCA, tricarboxylic acid.

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Bivalve mollusc culture seems to be a promising approach to meet both the VLC-PUFA and protein future demands. However, it comes with the barriers of food allergies and biotoxin hazard risks [9]. Other alternatives could be to harvest zooplankton, such as krill and copepods, but that has potential consequences on the marine environment if performed on a large scale [10]. A further promising approach could be the transfer of VLC-PUFA cluster genes from microorganisms to the crops commonly used for vegetable oil production [2]. However, this could be hindered by legal restrictions on the cultivation of genetically modified organisms, such as in Europe.

With the above-listed assumptions, the scientific community has focused on research into sustainable biomass production for VLC-PUFAs. Cultivation of VLC-PUFA rich microorganisms, such as marine protists (i.e. microalgae), is the most promising and viable solution to meet the current gap between VLC-PUFA supply and demand. This review thus critically discusses the availability and promises of the aquatic protists to be used in this application. Strategies to enhance sustainability and reduce the cost of the production process are also discussed. Moreover, a special focus on nutrient recycling from industrial food by-products and wastes using fermentation technologies is included, coupled with a biorefinery model to recover all high-value chemical compounds from biomass to achieve more sustainable production in the context of the circular economy.

Exploitation of metabolic biodiversity of aquatic protists for VLC-PUFA production

The term ‘protist’ generally refers to all unicellular eukaryotes, ranging from algae to heterotrophic flagellates, which are placed into a single kingdom of Protista [11]. However, ‘microalgae’ refers to a polyphyletic group of photosynthetic organisms, such as prokaryotic cyanobacteria and unicellular eukaryotes. Therefore, because the term ‘microalgae’ does not recognise that many protists can also grow heterotrophically or mixotrophically, currently protist is used to describe single-celled eukaryotes in general [12].

Many genera are obligate photoautotrophs, but some species can grow also mixotrophically such as *Brachiomonas*, *Chlorella*, *Chlorococcum*, *Cyclotella*, *Euglen*a, *Haematococcus*, *Nannochloropsis*, *Navicula*, *Nitzschia*, *Ochromonas*, *Phaeodactylum*, *Rhodomonas* and *Scenedesmus* [13]. Many of them are also facultative heterotrophs, belonging to genera *Amphora*, *Ankistrodesmus*, *Chlamydomonas*, *Chlorella*, *Chlorococcum*, *Cyclotella*, *Dunaliella*, *Euglena*, *Nannochloropsis*, *Nitzschia*, *Ochromonas* and *Tetraselmis* [13]. Moreover, some protists are obligate heterotrophs such as *Crypthecodinium* and thraustochytrids, but also many dinoflagellates such as *Oxyrrhis* and *Gyrodinium.*

Protists are important VLC-PUFA producers and, therefore, are considered possible candidates for industrial production of EPA and DHA. Those producing VLC-PUFAs belong mainly to marine phytoplankton [14]. However, some freshwater protists, such as *Monodus subterraneus* and *Trachydiscus minutus* are considered potential EPA-producers [15,16]. Generally, protists grown in heterotrophy and mixotrophy have increased VLC-PUFA content [17]. VLC-PUFA content and cultivation strategies of some genera of marine and freshwater protists are summarised in Table 1.

Among the obligate heterotrophic protists, it is reported that the phagotrophs *Ochromonas marina* and *Gyrodimium dominans* produce more EPA and DHA when fed on dried yeast [18], while thraustochytrids (*Aurantochytrium* spp., *Thraustochytrium* spp. and *Schizochytrium* spp.) and dinoflagellate *Crypthecodinium cohnii* are considered mainly DHA producers [19,20]. In particular, for thraustochytrids, a DHA content of more than one third of the total fatty acids is usually reported [20,21]. Moreover, *Schizochytrium* sp. is also used for the industrial production of DPA [22]. Mixotrophic growth has been reported to improve lipid productivity of many protists. In particular, *Nannochloropsis gaditana*, *N. oculata*, *Dunaliella salina and Chlorella sorokiniana* produce a higher amount of lipids in mixotrophy, compared with photoautotrophy [23,24].

Table 1
Reported cultivation strategies and average content of EPA, DHA and DPA as % of total fatty acids (TFA) from some genera of marine and freshwater protists.

Phylum	Genus	Cultivation type	VLC-PUFA (% of TFA)	Reference
Bacillariophyta	*Phaeodactylum*	P, M	18.6 EPA, 1.3 DHA	[16]
	Nitzschia	P, M, H	13.8 EPA, 1.1 DHA	[76]
	Skeletonema	P	10.9 EPA, 1.4 DHA	[76]
	Thalassiosira	P	15.1 EPA, 3.9 DHA	[14]
	Odontella	P	19.8 EPA, 2.9 DHA	[76]
	Cyclotella	P, M, H	15.4 EPA, 1.2 DHA	[76]
Ochrophyta	*Nannochloropsis*	P, M, H	21.0 EPA	[16]
	Chloridella	P	28.7 EPA	[16]
	Monodus	P, M, H	12.0 EPA, 2.3 DHA	[16]
	Trachydiscus	P	38.7 EPA	[15]
Rhodophyta	*Porphyridium*	P, M, H	16.7 EPA	[16]
Cryptophyta	*Rhodomonas*	P	10.7 EPA, 6.9 DHA	[76]
	Chroomonas	P	13.4 EPA, 4,7 DPA	[76]
Chlorophyta	*Tetraselmis*	P, M, H	9.5 EPA	[16]
	Koliella	P	5.2 EPA	[16]
Heterokonta -Bygira	*Aurantochytrium*	H	39 DHA	[77]
	Schizochytrium	H	43.1 DHA	[36]
	Thraustochytrium	H	69 DHA, 13 DPA	[77]
	Ulkenia	H	13.7 DHA 2.4 DPA	[77]
Haptophyta	*Emiliania*	P	19.7 DHA	[14]
	Isochrysis	P, M, H	1.9 EPA, 6.6 DPA, 14.3 DHA	[16]
	Pavlova	P, M	27.8 EPA, 6,6 DPA,6,6 DHA	[76]
Miozoa	*Amphidinium*	P, M	7.6 EPA, 2.6 DPA, 10.4 DHA	[16]
	Crypthecodinium	H	28.8 DHA	[19]
	Pyrocystis	P, M, H	24.3 EPA, 41.1 DPA	[16]
	Prorocentrum	P, M	24.1 EPA, 20.6 DHA	[16]
	Oxyrrhis	H	1.4 EPA, 15.3 DHA	[18]

Legend: P = photoautrophic, M = mixotrophic, H = heterotrophic cultivation.

Besides the regular metabolism, it is possible to enhance the lipid productivity of protists by appropriate strain selection and by inducing mutagenesis and/or genetic engineering [13,25]. A study has reported that mutants of *Nannochloropsis oculata* increased the levels of EPA after N-methyl-*N*-nitrosourea-induced mutagenesis [25]. In *Pavlova lutheri*, instead, after mutation by UV-light, the EPA and DHA content were 32.8 % and 32.9 % (as % dry biomass) respectively, higher than those of native strain [26]. Recently, an improvement in DHA and EPA content of *Schizochytrium* sp. by 81.5 % and 172.5 % respectively was reported, that could be of interest to apply at an industrial scale [27].

State of production technologies

The cultivation technology for aquatic protists represents a key point for VLC-PUFA production and improvement of lipid yields. Cultivation technologies are based largely on the metabolism of the species. Autotrophic cultivation is the oldest method to cultivate microalgae, and the main industrial technology used in autotrophy is the open pond system

[28]. Open ponds are built in a raceway or circular configuration; in the former, biomass surface is exposed to sunlight as much as possible and its movement is guaranteed by paddle wheels that provide regular mixing and recirculation, preventing biomass sedimentation; in the second configuration instead, the tank has a cylindrical shape and biomass is continuously stirred by a pivoted rotating agitator. However, due to several limitations, this configuration has been set aside and not used for industrial cultivation [29].

In order to prevent contamination and to control critical parameters such as CO_2 utilisation, light intensity and temperature, photobioreactors (PBRs) and indoor ponds are developed [30]. Nevertheless, the development of increasingly sophisticated PBRs with lower investment costs has been one of the main targets of recent years [28]. Closed PBRs allow control over all the growth parameters and avoid wasting CO_2. However, the high investment cost for these plants remains the main problem [30]. The classic PBR designs are the tubular (vertical and horizontal) and flat-panel systems. The tubular design is made with transparent tubing where the culture flows with a certain speed [31], while the flat panel reactors consist of two parallel panels (usually made in PVC) between which there is a layer where biomass grow [32]. Nevertheless, these designs have some disadvantages such as high investment costs, difficulty in light absorption and biomass harvesting and the absence of possibility to scale up in large-scale production [32,33]. To obtain a more uniform light distribution in tubular PBRs, an innovative design where the tubes are immersed in a suspension of light-scattering silica nanoparticles were designed [33]. This reactor was tested to grow *Chlamydomonas reinhardtii*, a protist rich in VLC-PUFAs.

Unlike autotrophy, heterotrophic conditions require the addition of an organic carbon source but not light. Thus, heterotrophic growth can be performed in conventional microbial bioreactors, reducing the initial investment costs. Recent studies have shown that the use of ‘closed’ biofermenters for the production of VLC-PUFAs, is the best method to produce these fatty acids [34]. Some engineering strategies have been established in the context of VLC-PUFA production from many protists, such as fed-batch fermentations. In fed-batch strategies, the amount of organic carbon is not supplied to the culture all at once but is spread out over time, depending on the metabolic rate of the species [35]. In fed-batch cultivation of *Schizochytrium* sp., enhancement of DHA production and doubling of lipid productivity, compared to batch cultivation methods, have been demonstrated [36]. However, fed-batch has the limitation of low volumetric productivity [35]. For that reason, a continuous cultivation mode (where the volume of bioreactors is constant) was also developed for PUFA-producer species. Different strategies to increase lipid productivity were developed, the most common of which relies on nitrogen starvation, that induces lipid accumulation, but causes a drop in biomass growth rate [37]. To overcome this limitation, an innovative multi-stage continuous cultivation was developed to obtain a good compromise between growth rate and relative amount of the target molecules such as VLC-PUFA and/or secondary metabolites [37,38]. In a recent study, a three stage approach has been developed for *Schizochytrium* sp. cultivation, obtaining an increase of lipids, DHA content and DHA productivity by 47.6 %, 64.3 % and 97.1 % respectively, in comparison with the two-stage fermentation process [38].

Mixotrophic cultivation technologies are similar to those used for autotrophy, with minor modifications. One of the main technological challenges for mixotrophy, is to design a cost-effective system ensuring axenicity (requires steam-sterilisation) and, at the same time, also providing natural or artificial light. In a recent study, flat-panel PBRs were used to test an industrial scale-up for *Chlorella vulgaris* in mixotrophic conditions, concluding that there is ample room for engineering improvements [39]. An interesting technological variant was proposed in another study for mixotrophic cultivation. In this work, the lipid production of a mutant *Scenedesmus* sp. Z-4 was enhanced with an ultrasonic treatment, that led to an improvement of enzyme activity, cell membrane permeability and substrate transportation [40]. The amount and type of organic carbon to be used in mixotrophic cultivation requires further studies to establish the correct combinations for each species and strain [23]. There are currently a large number of studies related to mixotrophic cultivation for *Chlorella* sp.*,* *Nannochloropsis* sp. and *P. tricornutum*, but very few for other species [13,17,23,39,41]. This aspect is a limiting factor for industrial scale-up, as the mixotrophy is still limited to a few species, and often to those that do not produce a good amount of VLC-PUFAs.

Biorefinery concept to enhance sustainability and lower production costs

Aquatic protists are an important source of high-value compounds, including those producing VLC-PUFA: chlorophyll, canthaxanthin, lutein and beta-carotene from *Nannochloropsis* sp. [42], fucoxanthin from *P. tricornutum* [43], exopolysaccharides and phycobiliproteins from *Porphyridium cruentum* [34,44], β-carotene and violaxanthin from *N. gaditana* [23], carotenoids from thraustochytrids [45] and astaxanthin from *Aurantiochytrium* sp. [46]. Therefore, one of the strategies to improve the sustainability of VLC-PUFA production by aquatic protists, is the exploitation of all the possible high-value co-products from the whole biomass and the residual spent medium for cultivation [47]. One of the possible downstream biorefinery approaches is shown in Fig. 1. Many molecules such as extracellular polymeric substances (EPS) are released during the cultivation process. Moreover, residual spent media after harvesting is rich in residual nutrients that could be recycled into the cultivation process, which is a common practice especially in autotrophic cultivation to lower production costs [48].

After lipid extraction, the residual defatted biomass (cake) can also be used to recover high-value compounds (i.e. phycobiliproteins) and/or used as protein-, carbohydrates- and mineral-rich biomass for feed supplementation [49]. The lowest value application of the residual cake could be anaerobic digestion for the recovery of energy and mineral nutrients in the production process. In a recent study, the defatted biomass was used as feedstock for the production of bio-hydrogen through anaerobic digestion and also to recover reducing sugars that were reused in the cultivation process [50]. Moreover, the process for lipid purification and omega-3 concentration requires the removal of high value pigments (i.e. carotenoids) and short chain fatty acids suitable for energy production into the biorefinery. On-site conversion for energy production often requires additional equipment, increasing capital costs; also, valorisation of co-products in other markets may have better economic sustainability [47].

Some omega-3 rich protists show a high content of light-harvesting pigments such as phycobiliprotein (PBP) that are widely used as natural dyes for food and cosmetics. *P. purpureum* has a total PBP content of 4.8 % on a dry basis consisting of 70 % phycoerythrin, 20 % C-phycocyanin and 10 % allo-phycocyanin, all pigments with high economic value [44]. In fact, a study estimated the total cost for highly purified PE production in *P. cruentum* at USD 1.17 mg^{-1}, while the commercial price of standard PE is higher than USD 30 mg^{-1} [51].

From the omega-3 crude oil, it is also possible to recover some high-value carotenoids. Fucoxanthin (FX) represents a major carotenoid in diatoms and presents several health benefits thanks to its claimed antioxidant, anti-inflammatory, anticancer, and antihypertensive activities [52]. Recently the FX production of 13 diatoms in photoautotrophy were studied, reporting the highest value in *Odontella aurita* ($>$0.20 mg $L^{-1}d^{-1}$) [53]. Another study obtained 5.97 mg L^{-1} of FX using *P. tricornutum* with a supplementation of spent yeast (versus 1.82 mg L^{-1} from the control) [43].

Thraustochytrids are reported to be a source of xanthophyll carotenoid astaxanthin. Astaxanthin productivity of 9.48 mg $L^{-1}d^{-1}$ was reported through the cultivation of *Aurantiochytrium sp.* mutant, with a yield of 40 mg L^{-1} [46]. Another study reported an astaxanthin yield of 162.14 μg g^{-1} from *Thraustochytrium sp. S7* optimized with response surface methodologies [54]. Astaxanthin and β-carotene represent

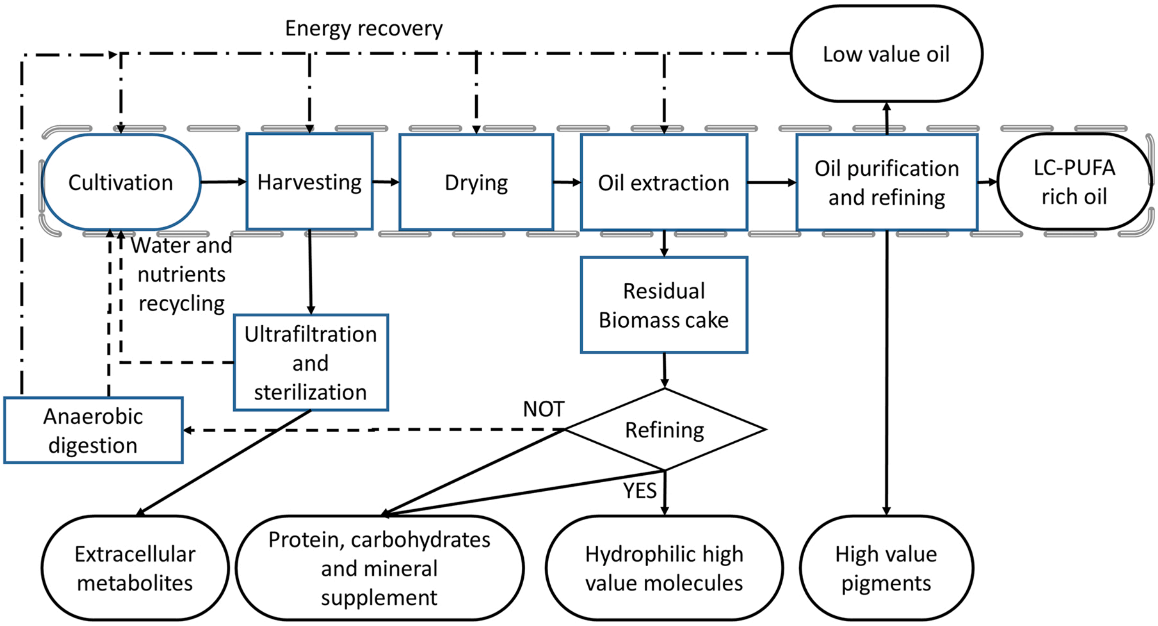

Fig. 1. Multiproduct biorefinery model for production of algal oil using as a platform a VLC-PUFA rich protist.

almost half of the global carotenoid market, which was estimated to be $1.2 billion in 2016 and is expected to increase to over $1.5 billion by 2021 [55]. Others postulated a biorefinery model for biofuel production that can also be implemented in the cultivation of PUFA-rich protists. The authors concluded that without an integrated approach, microalgal biodiesel could never be produced economically [56]. From the reported data, it is clear that a proper biorefinery design with a proper fractionation system as suggested in Fig. 1, can also be of environmental and economic profit for VLC-PUFA production.

Use of recycled nutrients for food-grade PUFA production

Despite the ability that many protists have to grow on wastewater and polluting substances, here we will evaluate only the use of food grade elements for the development of the biomass. From the perspective of a more economical cultivation process, heterotrophy has a great advantage; without the light requirement, heterotrophic protists can also grow in a dark coloured media or in the presence of suspended solids that make the passage of light difficult [57]. This advantage could be exploited also for mixotrophic cultivation.

Nutrients required during the cultivation of PUFA-rich microorganisms contribute significantly to the overall costs and carbon footprint of the final product. To overcome this limitation, the recycling of nutrients from agro-industrial flue gas, side-streams, waste and by-products seems to be one of the best approaches for VLC-PUFAs production from aquatic protists [58]. Although there are different sources of agro-industrial waste, those ones coming from the food industry can be reused in fermentation technologies to produce food-grade high value products. Food industry by-products and waste (FBW) are characterised by high amounts of organic carbon, proteins and mineral salts, which could be usefully recovered for biomass cultivation [59,60]. Moreover, these FBW are easily obtained, being produced in large quantities, particularly those from the agro-industry, for which an increase is expected in coming years [61].

To date, some FBW have been successfully used for the cultivation of microorganisms. Among them, the cheapest are sugar molasses, corn steep liquor (CSL), whey permeate (WP) and glycerol. These by-products are successfully used in the cultivation of aquatic protists rich in VLC-PUFAs; other available FBW such as brewery by-product and food waste showed promising results (Table 2). FBW usually needs pre-treatment before use as nutrients. Complex organic solids such as carbohydrates (starch, cellulose, lactose etc.), lipids and proteins must be hydrolysed to release basic components (i.e. monosaccharides and amino acids) that are easily usable by microorganisms [58]. The pre-treatments aim to a) remove particulates and reduce colouring effect for mixotrophic cultivation (avoiding light-shading effects); b) increase the bioavailability of organic compounds (i.e. particle size reduction, protein and carbohydrate hydrolysis); c) remove or reduce the number of toxic compounds; and d) increase stability of FBW and related carbon loss during transport and storage before use [62]. However, it is not well known if the pre-treatment of FBW could affect fatty acid production by aquatic protists, but the utilization of FBW in the culture media could induce changes in the biomass biochemical composition [60].

Different approaches to treatments for microalgal cultivation have been evaluated in the last few years, one such approach being enzymatic hydrolysis. Commercial amylolytic and proteolytic enzymes were used in submerged fermentations to treat food waste for *Chlorella pyrenoidosa*, obtaining a hydrolysate rich in glucose and free amino nitrogen (FAN) [63]. Fungal hydrolysis was reported also as an effective pre-treatment of food waste for heterotrophic cultivation of *Schizochytrium mangrovei* and *C. pyrenoidosa* [64]. Anaerobic digestion (AD) of agro-industrial wastes, wastewater and by-products coupled with microalgae cultivation is reported as a strategy to couple bioenergy production and nutrient recovery from liquid digestate rich in ammonia, phosphate and organic acids [65]. Liquid digestate from agro-industrial waste has also been used to cultivate EPA-rich diatom *P. tricornutum* [59].

All these studies suggest that FBWs are interesting sources of organic carbon, mineral salts and nitrogen. However, more in-depth and extensive research is required for sustainable FBW pre-treatments, selection of suitable strains and optimisation of culture conditions. In fact, it would be interesting to combine the concept of biorefinery explained above with the possibility of reusing low-cost nutritional sources to make VLC-PUFA production process economically and environmentally more sustainable.

Table 2
Aquatic protists rich in n-3 LC-PUFAs cultivated using agro-industrial by-products.

Food by-products	Species	Total lipids (% of biomass)	VLC-PUFA (% of TFA)	Biomass concentration (g L^{-1} DW)	Reference
Corn steep powder + glycerol	*Aurantiochytrium sp. n. AF0043*	31.14 %	DHA 29.7 % DPA 6.0 %	29.78	[78]
Cheese whey + Corn steep liquor	*Crypthecodinium cohnii* CCMP 316	28.7%	DHA 8.5 to 17 %	6.0	[79]
Tofu whey	*Schizochytrium sp.* S31	56.8 %	DHA 22.5 % DPA 3.9 % EPA 1.4 %	13.3	[75]
Carob pulp	*Crypthecodinium cohnii* CCMP 316	9.2 %	DHA 48 % DPA 2.1 %	42.0	[80]
Potato processing water	Thraustochytriidae sp. AS4-A1	38 %	DHA 28.9 %	9.0	[81]
Liquid residue from brewery by-product	Thraustochytriidae sp. AS4-A1	31 %	DHA 21.5 % DPA + EPA 21.5 %	9.01	[81]
Liquid residue from brewery by-product yeast extract + monosodium glutamate	Thraustochytriidae sp. AS4-A1	50 %	DHA 13.3 %	15.2	[81]
Saline water from demineralization of cheese whey + glycerol	*Schizochytrium limacinum*	35 %	DHA 48.46 %	28.40	[82]
Saline water from demineralization of cheese whey + yeast extract + glycerol	*Japonochytrium marinum*	51.47 %	DHA 48.98 %	24.72	[82]
Cane molasses	*Schizochytrium sp. CCTCC M209059*	41.22 %	DHA 37.9 % DPA 12.08 % EPA 1.16 %	21.94	[83]
Cane molasses + algae-residue	*Schizochytrium sp.*	32.8 %	DHA 45.26 %	55.54	[84]
High-fructose corn syrup	*Aurantiochytrium sp.* YLH70	64.9 %	DHA 39.41 %	78.5	[85]
Brewery spent yeast	*Aurantiochytrium sp.* KRS101	38.1 %	DHA 34.2 %	31.8	[86]
Brewery spent yeast	*Phaeodactylum tricornutum*	N.r.	EPA 16 %	0.8	[43]
Food waste (rice. noodles and breads) + glycerol + antioxidant	Phaeodactylum tricornutum engineered strain E70	35 %	ARA 5.6 % DHA 3.3 % EPA 25.9 %	N.r.	[87]

N.r.= Not reported

Economy and sustainability

The so-called “omega-3 algae oils” are considered niche products in the market [13]. Very few companies have the production platform in place for it and mainly use thraustochytrids in closed bioreactors, and only a handful of companies sell food-grade omega-3 oil made using a photosynthetic technology (Table 3). Most of the photosynthetic production plants for omega-3 rich microalgae have the niche specialties for aquaculture as a core market. However, the VLC-PUFA market price is predicted to grow at an average annual rate of 13.5 % worldwide,

Table 3
Main companies producing VLC-PUFA oils from protists. Information provided by direct mail interviews with some of the companies and company websites.

Company – registered trademark	Product	Strains used	Cultivation technology	Carbon source	Production area	Ref.
DSM - VERAMARIS®	Omega 3 algae oil	*Schizochytrium sp.*	Biofermenters	Dextrose from corn	Netherlands	[88]
DSM – Martek Biosciences	Omega 3 algae oil	*Schizochytrium sp.*	Biofermenters	N.r.	N.r.	[89]
ALGORIGIN®	Omega 3 capsules	*Schizochytrium sp.*	Biofermenters	N.r.	England	INW - [90]
Goerlich-Pharma - BIOPLUS	Algae oil Capsules Algal oil Powder	*Schizochytrium sp.*	Biofermenters	N.r.	Germany	[91]
CELLANA	Omega 3 for feed and nutraceutical	Various marine strains (unknown)	Phototrophic open pond – PBR combination	Carbon dioxide	USA	[92]
LYXIA®	Algae oil bulk Algae oil powder	*Nannochloropsis salina* *Schizochytrium sp.*	Phototrophic open raceway pond -Biofermenters	Carbon dioxide	China	[93]
Qualitas Health - IWI LIFE®	Omega 3 capsules	*Nannochlorpsis sp.*	Phototrophic open raceway pond	Carbon dioxide	USA - Mexico	[94]
Source-Omega	Algae oil	*Schizochytrium sp.*	Biofermenters	Sugars from corn industry	USA	[95]
Corbion - AlgaPrime™ DHA	Omega 3 for aquaculture, pet food and livestock	*Schizochytrium sp.*	Biofermenters	Sugar from sugar cane	Brazil	INW - [96]
FERMENTALG	Omega 3 for nutraceuticals DHA oil	Various strains (*Ulkenia sp.*, *Schizochytrium sp.*)	Biofermenters	N.r.	France	[97]
Chambio – ALGAMEG-3	Algal oil powder	*Schizochytrium* AlgaMEG-3TM	Biofermenters	N.r.	Taiwan	[98]
Algarithm	Algal oil – oil powder	*Schizochytrium sp.*	Biofermenters	N.r.	Canada	[99]
Algaenutra	Algal oil – oil powder	*Schizochytrium sp.*	Biofermenters	N.r.	China	[100]
Arizona Algae Products – EPA15+	EPA omega 3 algae oil	*Nannochloropsis WPRO30+*	Closed Photobioreactors and covered raceway	Carbon dioxide	Arizona, USA	INW - [101]
Mara Renewables Corporation	DHA omega 3 algae oil	*Schizochytrium T18*	Biofermenters	Glucose	UK	INW - [102]

N.r. = Not reported; INW = interview to producers; Numbers refer to web page of producers.

reaching a value of \$5 billion in 2020 [66]. The market supply is ensured by fish oil and a fluctuating value of about 1 million metric tons of fish oil per annum is reported from whole fish and fishery by-products from 2015 to 2018, with a mean price of \$1600 ton^{-1} [67]. The main destination for fish oil is the aquaculture sector; other markets include terrestrial animal feed, direct human consumption and other special uses. The Global Organization for EPA and DHA reported a total share of 111,210 metric tons of EPA and DHA ingredients in 2018, of which about 2000 tons were algae oil [68]. In terms of volume, dietary supplements are the market leader (63.8 %) followed by pet food supplementation (24.8 %), infant formulas (4 %) and the remaining are fortified foods and pharma products [67].

For a view of the final market of omega-3 from supplements and other foods, an interesting study reports an evaluation of the unit price of EPA-DHA in some products available in supermarkets [69]. The lowest economic value was observed for cheap fish oil with an EPA-DHA price of \$60 kg^{-1}. Instead, a price of \$180 was reported for 1 kg of EPA-DHA from frozen sardine, while prenatal DHA and nutraceutical omega-3 supplements showed a cost range of \$870 to 2500 kg^{-1}.

The literature is scarce regarding the analysis of production costs and life cycle assessment of aquatic protists cultivation for VLC-PUFA production, but many authors have reported data on biodiesel production. For phototrophic cultivation, one study lists the main factors for lowering production costs as: the biological productivity of the microalgal strain, the photosynthetic efficiency of the cultivation system and geographical location which influences solar irradiation and temperature, and access to cooling water for PBRs [70]. It was concluded that using microalgae with 6% of their biomass consisting of EPA and DHA, cultivated in flat panel PBRs in Spain, have the lowest production cost (\$39 kg^{-1} of EPA/DHA equivalents) with respect to the use of tubular systems and open pond raceways.

Another study reported on *Tetraselmis suecica* cultivated in PBRs, a biomass cost of \$14 kg^{-1} at 1-ha scale, modelling a cost of \$5.7 kg^{-1} for 100-ha. However, it was concluded that locating the plant in more favorable climatic conditions (e.g. in Tunisia), the final cost of the biomass could be reduced by up to \$3.6 kg^{-1} at the 100-ha scale [71]. Others have reported, in a techno-economic analysis of heterotrophic biofuel production using *C. protothecoides*, that for a plant producing 10.126 ton yr^{-1} biodiesel the production cost was \$1.224 ton^{-1}. It was stated that the investment was not profitable for biofuel alone but it should improve if the biomass were sold at a high price and a technology that is less energy intensive used to harvest, break the cell wall and to extract the oil [72]. This could be the case in a factory dedicated to the omega-3 oils.

Generally, 20–30 % of the total cost of biomass production is represented by biomass harvesting, while the equipment cost for the extraction/esterification of oil from biomass is 6 % of the total equipment cost [73]. The conventional method for lipid extraction involves the use of organic solvents, but first, a suitable cell disruption must be conducted to extract lipids. In order to increase extraction yields, novel techniques have been developed to aid cell wall disruption. These techniques are principally ultrasonic assisted extraction (UAE), microwave assisted extraction (MAE) and supercritical fluid extraction (SCF), which are also used on an industrial scale [72].

Another important cost in mixotrophic and heterotrophic cultivation is linked to the use of organic substrates. Using pure chemicals as a carbon source is not feasible for large scale operation if the aim is to compete with the reference market of the final product (omega-3 from fish in the present case). It is estimated that the glucose represents about 80 % of the total medium cost, so that using by-products can cut down the costs [74]. The organic carbon and nitrogen substrates should be supplied from by-products of other processes to overcome this limitation [72].

A recent study reported a production cost for DHA produced by *Schizochytrium sp.* S31 using standard media in the range of \$52.2–157.2 kg^{-1}, while a further improvement of the process using a sustainable medium reduced it to \$15.4 kg^{-1} [75]. Another report using laboratory results based on oil and high-value pigments produced by *Nannochloropsis sp.* in indoor polyethylene bag PBRs, found that 82 % of the costs were associated with light, 13 % with water, 4 % with nutrient consumption and an unusual 1 % with harvesting [42]. Data from these later works suffer from not considering labor, equipment, land investments and indirect costs.

Conclusion

Aquatic protists can be used effectively for the industrial production of long chain omega-3 for human consumption. Quality, safety and ethical issues related to this oil generate consumer motivation to pay more than they would do for fish oil. However, for protists to emerge from the niche market of vegan supplements and establish in the massive food market, some steps in research and development are required to meet economic and environmental sustainability standards. First, screening and selection of wild-type and mutant strains are required to identify the species with the highest EPA and DHA productivity. Secondly, optimisation of cultivation protocols and technologies, utilisation of agro-food by-products as low-cost nutrients for media formulation and recovery of high-value co-products from the residual biomass in a biorefinery concept must all be explored to improve sustainability and meet the promise of protist cultivation as an alternative source of VLC-PUFAs.

Author contributions

GLR and ALL designed and contribute to the review equally; GLR and MO collected and summarized the references and data; GLR, ALL, RS, PM revised the text; GLR, RS, PM and MO edited it.

Declaration of Competing Interest

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

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