

Omega-3/6 fatty acids: Alternative sources of production

Owen P. Ward^{*}, Ajay Singh

Department of Biology, University of Waterloo, Waterloo, Ont., Canada N2L 3G1

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Abstract

Polyunsaturated fatty acids (PUFAs) are essential components of higher eukaryotes. Single cell oils (SCO) are now widely accepted in the market place and there is a growing awareness of the health benefits of PUFAs, such as γ -linolenic acid (GLA), arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). ARA and DHA have also been used for fortification of infant formulae in many parts of the world. Fish oils are rich sources of DHA and EPA and a limited number of plant oilseeds are good sources of other PUFAs. Marine protists and dinoflagellates, such as species of *Thraustochytrium*, *Schizochytrium* and *Cryptocodinium* are the rich sources of DHA, whereas microalgae like *Phaeodactylum* and *Monodus* are good sources of EPA. Species of lower fungi *Mortierella* accumulate a high percentage of ARA in the lipid fraction. In this paper, various microbiological and enzymatic methods for synthesis of PUFAs are discussed. © 2005 Elsevier Ltd. All rights reserved.

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1. Introduction

Polyunsaturated fatty acids (PUFAs) are essential components of higher eukaryotes. They confer flexibility, fluidity and selective permeability properties to membranes. The brain is particularly rich in arachidonic acid (ARA) and docosahexaenoic acid (DHA), and the latter is also a ligand for the retinoid X-receptor [1]. Eicosapentaenoic acid (EPA) has a beneficial effect on the cardiovascular system. PUFAs contained in membrane phospholipids (PL) are precursors for synthesis of prostaglandins, leukotrienes and thromboxanes which bind to specific G-protein-coupled receptors and signal cellular physiological responses to inflammation, vasodilation, blood pressure, pain and fever [2]. Consequently, PUFAs and their derivatives and analogues are important nutraceutical and pharmaceutical targets [3].

In 1989, we published a review in this Journal on “Omega-3 Fatty Acids: Alternative Sources of Production” [4]. Many exciting developments have occurred in this field in the past 15 years. This review attempts to update these

developments. Single cell oils (SCO) are now widely accepted in the market place and there is a growing awareness of the health benefits of PUFAs, such that the market for specific products is predicted to expand and diversify. In addition, developments in processes for PUFA production are benefiting from advances in cell and molecular biology and recombinant technology. The major targets so far have been γ -linolenic acid (GLA), ARA, DHA and EPA. While the term highly unsaturated fatty acids (HUFAs) is sometimes used to differentiate higher from lower forms of PUFAs, in most cases we have retained the term PUFA.

Strategies to develop processes for production of single cell oil should take account of lessons learned from attempts to commercialize processes for production of single cell proteins (SCP). During the period from 1960 to the mid-1980s there was substantial activity in development and commercialization of processes to produce proteins from microorganisms as a source of food/feed for humans and animals. The general strategy to produce SCP by fermentation failed dismally because of the lower cost of plant proteins, the stability of agricultural prices relative to industrial prices and the low value of food protein and particularly feed protein. Use of cheap hydrocarbon-based

^{*} Corresponding author. Tel.: +1 519 888 4567x2427;
fax: +1 519 746 0614.

E-mail address: opward@sciborg.uwaterloo.ca (O.P. Ward).

nutrients in the fermentation medium also proved problematic because it resulted in a need for more complex downstream processing methods to separate the proteins from residual toxic hydrocarbon contaminants.

Single cell oil production was explored in the 1980s for production of cocoa butter substitutes since cocoa butter was in short supply [5]. Selected yeasts, which had a fatty acid profile similar to that of cocoa butter (a triacylglycerol with approximately equal amounts of stearate, oleate and palmitate) were used to produce cocoa butter equivalents. Candidate yeasts were *Rhodospiridium toruloides* and *Cryptococcus curvatus*. The most effective approach involved partially blocking the Δ -9 desaturase (converts stearate to oleate) by mutation of *C. curvatus* to increase the amount of stearate (typically less than 10%, w/w) at the expense of oleate. The resulting SCO contained 16:0–18:0–18:1 in the ratio 24:31:30 (% w/w), which is quite similar to cocoa butter (28:35:35) [6–9]. But even this example of a higher value oil exhibited a somewhat similar history to SCP developments [10], when the world price of cocoa butter dropped from \$8000/tonne to <\$2500 [11]. There are predictions that there is likely to be a shortfall in production of cocoa beans by 2004, so the price of cocoa butter may rise, again generating interest in production of cocoa butter equivalents. However, one should be cautious about predicting the future commercial success of this process as there may also be other competitive approaches. For example, olive oil or palm oil may be converted to cocoa butter equivalents by increasing the stearic acid content by lipase transesterification in hexane media [12]. Lipase-mediated enrichment of plant oils with omega-3 fatty acids is discussed in Section 14.

The principal lesson to be learned from both the SCP and cocoa butter substitute experiences is that the strategy for single cell oil production rightly needs to target higher value materials and the main focus on single cell oil technology development is on production of long chain PUFAs with applications in human health, as nutraceuticals, pharmaceuticals and pharmaceutical precursors. And even in this area there is a need to continually evaluate the potential for alternative approaches to synthesis of these products to emerge, specifically through plant biotechnology and through transformations of more available fatty acid species mediated by enzymatic or chemical methods or their combinations

2. Health related aspects of PUFAs

DHA is a major structural component of the gray matter of the brain and the eye retina and an important component of heart tissue. As a result dietary DHA has been shown to be important for proper development of the brain and eye in infants and supports good cardiovascular health. ARA is the most abundant PUFA in humans, present in organs, muscle and blood tissue and has a major role as a structural lipid associated predominantly with PLs. ARA is the principal omega-6 fatty acid in the brain, and together with DHA, is important in the brain development of infants. While GLA is a metabolic precursor to ARA, its conversion to ARA, mediated by the enzyme Δ -6 desaturase, is slow and this enzyme is present only in low levels in humans. Hence, it is considered preferable to feed ARA to humans rather than GLA. ARA is also a direct precursor of a number of eicosanoids regulating lipoprotein metabolism, blood

Table 1
Milestone advisories related to use of DHA, EPA and ARA as dietary supplements

FAO and WHO recommend that infant formula should mimic breast milk	1975
Menhaden oil has Generally Recognized as Safe (GRAS) status from US FDA as a source of PUFAs for use in certain adult foods	1987
European Society of Pediatric Gastroenterology recommends that infant formula, both pre-term and term, should include ARA and DHA	1991
The British Nutrition Foundation recommends that infant formula, both pre-term and term, should include ARA and DHA	1992
A joint expert committee of FAO and WHO recommends that infant formula, both pre-term and term, should include ARA and DHA	1993
A successful regulatory review was completed in by the Ministry of Health in Holland which allowed infant formula makers to use specific commercial supplements of DHA and ARA in infant formulae	1995
An independent panel of experts in the US concluded that specific ARA and DHA supplements could be considered as GRAS for use in pre-term and term infant formulas by adults	1995
An independent panel of experts in the US concluded that a specific DHA oil could be considered as GRAS for use by adults, including pregnant and nursing women	1996
A meeting sponsored by the NIH and the International Society for the Study of Fatty Acids and Lipids in Washington DC recommended that infant formula be supplemented with DHA and ARA	1999
A Child Health Foundation panel recommended in Acta Paediatrica that infant formula contain both DHA and ARA	2001
The Canadian Government's Health Canada completed a favourable review of a submission supporting use of DHASCO and ARASCO oils in infant formulas in Canada	2003

Table 2
PUFA product applications and their sources

Product	PUFA	Plant	Fish	Microbe	Comment
Infant formula, term/pre-term	DHA, ARA			+	Low in EPA
Term infant formula	GLA	+			
Pregnant/nursing women	DHA			+	Low in EPA
Adult diet supplement	DHA, EPA		+		
	DHA			+	<i>Schizochytrium</i>
	ARA				
Food additive: cheese, yoghurt, spreads, dressings, breakfast cereals	DHA			+	<i>Schizochytrium</i>
Eggs	DHA	+			From flax
Mariculture	DHA, EPA		+		
	DHA, DPA				<i>Schizochytrium</i>
Pharmaceutical precursor	ARA			+	<i>Mortierella</i>
Cardiovascular health	DHA, EPA		+		
	DHA			+	
Atherosclerosis, hyperlipemia	EPA		+		Ethyl ester in Japan
Atopic eczema, rheumatoid arthritis, multiple sclerosis, schizophrenia, premenstrual tension relief	GLA	+			
Schizophrenia; certain cancers	EPA		+		

rheology, leucocyte function and platelet activation. Good nutritional sources of ARA are animal livers and egg yolks. While use of long chain PUFAs, to overcome cardiac problems, has long been advocated, concerns remain that EPA may contribute to thinning of artery walls in certain individuals which may cause serious bleeding problems. However, recent reports point to possible new applications of EPA, in treatment of brain disorders including schizophrenia [13,14] and for certain cancer patient conditions [15], may generate momentum for introduction of an EPA single cell oil production process.

Health and nutritional advisories on the use of highly unsaturated fatty acids date back to 1975 when the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) recommended that infant formula should mimic breast milk. With particular reference to PUFAs, human breast milk is rich in ARA and DHA [16]. From 1990 onwards a number of health and nutrition organizations specifically recommended inclusion of ARA and DHA in pre-term and term infant formula. Likewise the Food and Drug Administration (FDA) in the United States conferred Generally Regarded as Safe (GRAS) status on a

Table 3
Safety/performance aspects of PUFAs^a

Instability of DHA-containing oils from <i>Schizochytrium</i> and fish to oxidation limits the use of PUFA oils in processed foods and as nutritional supplements
No reports that <i>Schizochytrium</i> is pathogenic
No reports that <i>Schizochytrium</i> produces toxic chemicals and common algal toxins, such as domoic acid and prynesium toxin are absent
<i>Schizochytrium</i> spray dried microalgae used in aquaculture for 9 years as excellent stable dietary source of DHA for shrimp larva culture and finfish
Dried <i>Schizochytrium</i> microalgae has GRAS status for use as DHA-rich ingredient in broiler chickens and laying hen feed
Thraustochytrids especially, <i>Schizochytrium</i> are consumed directly by humans through consumption of mussels and clams
Up to 3 g DHA + EPA in Menhaden oil is safe
Fish oils improve reserves of DHA/EPA in pregnant/nursing mothers, low EPA fish oils are advised to avoid risk of bleeding
Ethyl ester of EPA has been used for treatment of atherosclerosis and hyperlipemia since 1990 in Japan
DHA in tuna oil has been used as an ingredient in infant formula and food
ARA rich oil from <i>Mortierella</i> has been incorporated into formula for pre-term infants in Europe
GLA, a precursor of ARA is effective in treatment of atopic eczema, rheumatoid arthritis, multiple sclerosis, schizophrenia and pre-menstrual syndrome
The fishy odor of fish oils limits their use as a source of EPA and DHA
GLA is used as an ingredient in infant formula and health food
ARA is an essential fatty acid and a precursor for biologically active prostaglandins and leukotrienes which have important roles in the circulatory and central nervous systems
DHA-45, from multi-step fermentation and refining process from a non-pathogenic, non-toxicogenic species of the <i>Ulkenia</i>
DHA-45 is reported to meet food grade and QA specs suitable for a refined edible oil intended as a food ingredient
DHA from <i>Cryptocodinium</i> available worldwide, e.g. Europe, Australia, Asia in pre-term and full-term formulas
A dried biomass from several algae is marketed as a source of EPA
Young children and pregnant and lactating females should avoid substantial fish oil consumption because of the risk of potential increased mercury intoxication or from PCBs, dioxins or other environmental contaminants with long half lives that can bioaccumulate in oils. These risks have caused the EPA and FDA to recommend limitations to fish consumption, specifying amounts and fish types. In general health related benefits to other groups far outweigh the risks

^a Compiled from [125–131].

variety of PUFA-containing products [17], for example, inclusion of menhaden oil in certain adult foods (1987), addition of certain microbial ARA/DHA-containing oils to pre-term and term infant formula, consumption of a microbial DHA-containing oil by pregnant and nursing women and use of a dried DHA-rich *Schizochytrium* preparation as a poultry feed [18,19]. The advisories are summarized in Table 1. ARA may be of particular benefit to vegetarian breast feeding mothers [20]. ARA may also be used as a synthon/pharmaceutical precursor. DHA and EPA have been used for general maintenance of good cardiovascular health. EPA has been used specifically for treatment of atherosclerosis and hyperlipemia, schizophrenia and certain cancers. GLA, from plant oils, has been incorporated into infant term formula and for treatment of conditions ranging from atopic eczema, rheumatoid arthritis, multiple sclerosis and premenstrual tension. GLA is present in oils from a limited number of plant oil seeds, for example, evening primrose (8–10%, w/w) and borage or starflower (20–23%, w/w) oils. PUFA product applications and their sources are summarized in Table 2.

Some of the safety and/or performance aspects of PUFA-containing fish oils or microbial oils are indicated in Table 3. Fish oil has been used as a source of DHA and EPA, although the fishy odor has been considered a disadvantage. For pregnant and nursing mothers and for infant formula, EPA content should be minimal because of its capacity to cause bleeding. While substantial consumption of certain fish or their oils by young children and by pregnant and nursing females is not recommended because of the potential for the oils to accumulate hazardous hydrophobic environmental contaminants, the health benefits of fish to other groups are considered to outweigh the risks. Major microbial sources of DHA (*Schizochytrium*, *Ulkenia* and *Cryptocodinium* spp.) and ARA (*Mortierella* spp.) are considered to be non-pathogenic and non-toxicogenic. DHA from *Cryptocodinium*

Table 4

Possible mechanisms by which omega-3 fatty acids reduce risk of cardiovascular disease

Susceptibility of heart to ventricular arrhythmia reduced
Fasting and postprandial hypoglycemic effects
Mild hypotensive effects
Nitric oxide induced endothelial relaxation promoted
Retardation of atherosclerotic plaque growth
Reduced adhesion, platelet derived growth factor
Anti-inflammatory properties

has been used on a worldwide basis in infant formula and *Mortierella* ARA has been incorporated into pre-term infant formula in Europe. There is a general concern regarding the oxidative instability of PUFA-containing oils. In the case of direct consumption of PUFA-containing oils this is overcome through use of capsules while microencapsulation techniques can address this problem for incorporation of oils into dried foods.

Attention to the health benefits of PUFAs first emerged when it was noted that populations deriving a substantial proportion of their food from fish had a much lower incidence of heart disease. The mechanisms by which omega-3 fatty acids reduce risk of cardiovascular disease are well established (Table 4). It is estimated that such populations consume 0.5–0.7 g/day DHA whereas DHA and EPA account for only 0.1–0.2 g/day in the US diet. The typical 'Western' diet, which provides for high levels of omega-6 PUFAs and low levels of omega-3 PUFAs is considered to be imbalanced. General recommendations for daily dietary intakes of DHA/EPA are: 0.5 g for infants, an average of 1 g/day for adults and patients with coronary heart disease (CHD) and 2–4 g/day for management of hyperglyceridemia with medical consultation [21]. Indicators or recommendations of daily dietary intakes of PUFAs are provided in Table 5 together with some small or moderated side effects of doses at the higher end. As is

Table 5

Omega-3 fatty acid intake aspects^a

Consumption of omega-3 fatty acids in the US is approximately 1.6 g/day, predominantly as ALA (1.4 g/L) with some DHA and EPA (0.1–0.2 g/L).

The major sources of ALA are vegetable oils (such as canola and soybean) with other sources being flaxseed oil, and English walnuts

A total intake of ALA of 1.5–3 g/day appears to be beneficial

Fish are major sources of EPA and DHA, quantity varies with type, environment and whether wild or farm raised, see Table 6

Populations living on a large proportion of a marine diet have DHA intakes from 0.5–0.7 g/day

Health and Welfare Canada recommends daily intake of DHA/EPA to be a minimum of 0.22 g/day with a combined intake of 0.65/day

British National Foundation recommends females and males have an intake of DHA/EPA of 1.1 and 1.4 g, respectively

United States Institute of Medicine (IOM) recommends 0.5 g omega-3 PUFAs including DHA for infants

IOM recommends AIs of 1.6 and 1.1 g/day for ALA for males and females and that up to 10% can come from EPA/DHA

WHO recommended inclusion of DHA in infant formula

The American Heart Association encourages patients with CHD to increase their EPA/DHA consumption to 1 g/day through fish or dietary supplement consumption.

Typical 'Western' diet contains high omega-6 and low omega-3 PUFAs and is considered to be imbalanced

For medical management of hyperglyceridemia, larger omega-3 doses (2–4 g/day) may be administered in consultation with a physician in the form of high quality supplements

The following moderate side effects from ingestion of omega-3 fatty acids may occur at the daily dietary rates indicated: gastrointestinal upset,

1–3 g; fishy aftertaste, 1–3 g; worsening glycemia in patients with impaired glucose tolerance and diabetes, >3 g; rise in LDL-C in

patients with hypertriglyceridemia, 1–3 g

There is a low chance of clinical bleeding at dietary rates >3 g/day

^a Compiled from [21,126,128,132,133].

Table 6
Requirement of EPA and DHA per day from fish and other seafood diet

Fish/seafood diet required to provide 1 g of EPA + DHA per day	Grams per day
Fresh tuna	70–360
Sardines	60–90
Salmon	60–135
Mackerel	60–250
Herring	45–60
Rainbow trout	90–105
Halibut	90–225
Cod	375–750
Haddock	450
Catfish	450–600
Flounder/sole	210
Oyster (pacific/eastern/farmed)	75/195/240
Lobster	225–1275
Crab, Alaskan King	255
Shrimp	330
Clam	375
Scallop	525

illustrated in Table 6 consumption of a substantial amount of fish is required to maintain a daily dietary intake of 1 g DHA/EPA and hence the interest in obtaining these components from fish oil concentrates and microbial oils.

3. Markets for single cell oils

The world wholesale market for infant formula is estimated to be about \$10 billion per annum. ARA and DHA have been used for fortification of infant formulae in many parts of the world [22]. A range of companies have been marketing term infant formula and pre-term infant formula, containing single cell oils, in more than 30 and 60 countries worldwide, respectively. Some infant formulae marketed outside the US contain DHA from fish oil or omega-3 containing-eggs. Martek's ARA and algal DHA have received clearance for inclusion in infant formulae in the US. The DHA for this application (DHASCO), comes from the alga *Cryptocodinium cohnii*, and contains 40–50% DHA but no EPA or other long chain PUFAs. The ARA (ARASCO) comes from *Mortierella alpina* and contains 40–50% ARA and minimal amounts of other long chain PUFAs. It has been reported that Abbott Laboratories has requested FDA to give its DHA from fish oils and its fungal ARA GRAS status. Inclusion of microbial single cell oils in infant formula can add 10–20% to the retail price. As infant formula production is very price competitive, addition of this price premium may retard somewhat market acceptability of the SCO-supplemented product, especially given that experts differ on the efficacy of supplementing infant formulae with SCO.

The high DHA yields/productivities obtained with *Schizochytrium* species results in a production of a low cost oil which is used as adult dietary supplement in food and beverage markets, in health foods, in animal feeds and in

mariculture. Example foods are cheeses, yogurts, spreads and dressings, and breakfast cereals. Other markets include foods for pregnant and nursing women and applications in cardiovascular health. These markets may have much greater growth potential than infant formulae. Single cell oils are very sensitive to oxidation and considered not to be very compatible for direct incorporation into most liquid and dry foods on the market. Hence, finding methods which insure the stability of these oils in food and beverage applications is considered essential to effective expansion of the uses of SCOs in these markets. One method already introduced into commercial practice involves microencapsulation of the oil.

Because of the large potential size of the SCO market many companies have indicated their interest by developing processes, entering into licensing arrangements, manufacturing product, marketing products, patenting intellectual property or challenging granted patents. In addition, a key industry rationalization occurred when Martek acquired Omegatech, which at that time was Martek's main competitive producer of DHA-containing algal-derived oils. A partial list of companies researching, developing, manufacturing or marketing PUFAs or PUFA-containing products is presented in Table 7.

Martek has recently turned its research and development attention to cell genomics technology aimed at producing DHA in oil seed crops which it is hoped will substantially reduce the cost of DHA-containing oils. While this approach will likely, in the medium term, provide DHA or other omega-3-containing plants or plant oils as a new dietary source of these PUFAs, it is likely that microbial sources will be relied upon for purer preparations of individual PUFAs for infant formula, many dietary supplements and pharmaceuticals or their precursors. Mariculture applications of

Table 7
Partial list of companies reported to be researching, developing, manufacturing or marketing SCO PUFAs or PUFA-containing products

Aventis S.A.
BASF A.G.
Friesland Brands A.G.
Gist-brocades
Heinz-Wattie's
Hoffmann-LaRoche A.G.
Jamieson
Laboratorios Ordesa
Maarbarot
Martek Inc.
Mead Johnson Nutritionals
Nagase and Co.
Nestle S.A.
Novartis
Nutricia
Nutrinova Celanese A.G.
Pronova
Ross Products (Div of Abbott)
Suntory Ltd.
Walmart
Wyeth

microbial oils, in particular for feeding larval shrimp, brine shrimp, rotifers and mollusks require biomass aggregates of less than 50–150 μ . A spray dried preparation of *Schizochytrium* microalga has been used in aquaculture applications to enrich the latter species with DHA [23]. DPA is present in these formulations at a rate of about 10%. *Schizochytrium* species are consumed directly by man through consumption of mussels and clams.

4. Fermentation systems for production of PUFAs

A number of algal groups, including diatoms, crysophytes, cryptophytes, dinoflagellates and others, produce long chain PUFAs [24,25]. Algae that have been proposed for EPA production include *Nitzschia* spp. [26]; *Nannochloropsis* species [27]; *Navicula* species [28], *Phaeodactylum* species [4] and *Porphyridium* species [29]. Most algae are disadvantageous in that large amounts of EPA are not accumulated as triglycerides (TGs) and those that do accumulate TGs are obligate phototrophs [30]. For example, *Porphyridium cruentum* ARA and omega-3 fatty acids of *Phaeodactylum tricornutum* are associated with galactolipids, complex polar lipids not present in breast milk [4,31]. However, heterotrophic algae, such as *Pythium*, *Cryptocodinium* and *Nitzschia* species produce PUFAs predominantly as TGs and PLs.

Photobioreactors provide the ability to optimise culture parameters for algal growth and product formation. However, even with technologically advanced bioreactors for algal fermentations, maximum densities attained are low, making such processes cost-prohibitive for production of industrial products. Miron et al. [32] concluded that horizontal tubular photobioreactor technology was not realistic as a system for production of EPA by *P. tricornutum*. Although photobioreactors are prohibitively expensive to operate, photosynthetic algae will continue to be considered as candidate hosts for production of SCOs [33], especially for aquaculture where they likely represent the most natural and best source of a range of nutrients for larvae and fish fry.

An alternative to photobioreactors, with potential to reduce costs, is to use heterotrophic algae, grown in conventional fermenters [30]. With heterotrophs, photosynthesis for carbon and energy generation is replaced by supply of glucose or other utilizable carbon source to the medium. As a result, fermentation systems have been developed producing biomass densities of greater than 200 g biomass dry weight per litre of culture. Gladue and Maxey [34] estimated the cost of production of heterotrophic biomass to be <\$5/kg whereas production of a kg of algae phototrophically was estimated to cost 1–2 orders of magnitude higher [35,36].

Heterotrophic algae used in aquaculture include *Chlorella*, *Nitzschia*, *Cyclotella* and *Tetraselmis* species [34,37]. Zaslavskaja et al. [38] reported that the photoautotrophic

microalga, *P. tricornutum*, could be converted to a heterotroph by genetic engineering. Introduction of a single gene, encoding a glucose transporter enabled the organism to grow on glucose in the absence of light, opening up the potential to grow these organisms in high density culture and to overcome the limitations associated with light dependent growth. Much research has focused on the importance of PUFAs, particularly ARA, DHA and EPA, in larval growth and development and attention has specifically been directed towards *Schizochytrium* and related species, and to *Cryptocodinium* species, and indeed strategies have been devised to produce the marine fungal high DHA-producing *Schizochytrium* species in aggregates small enough to be consumed by larvae.

Originally PUFAs were considered to be absent from bacterial membranes [39]. However, many bacterial species of marine origin, particularly species found in high pressure and low temperature deep sea environments, have been shown to produce PUFAs, such as EPA and DHA [40–42]. Several marine bacteria contain EPA and DHA in an amount of up to 25% of total membrane fatty acids [43]. The requirement of many of these species that they be cultivated at low temperatures and at high pressures makes them unattractive candidates as commercial PUFA production strains. However, these PUFA-producing strains are important from the perspective of understanding their biochemical and genetic mechanisms for PUFA synthesis and as potential sources of genetic material for transfer to more suitable potential industrial hosts. Bacterial strains, whose PUFA production systems have been characterized, include *Photobacterium profundum* strain ss9 [44], *Shewanella* strain SCRC-2738 [45] and *Moritella marina* strain MP-1 (formerly *Vibrio marinus*) [46].

5. Microbial production of GLA

An overview of process investigations related to microbial production of GLA is presented in Table 8. Researchers have demonstrated the potential to produce GLA at concentrations of 15–25% of total fatty acids (TFAs) in oils produced by lower fungi from the order Mucorales, especially from *Mortierella*, *Mucor* and *Cunninghamella* species. It is possible to achieve biomass densities of >50 g/L in submerged culture in culture periods of typically 10 days with productivities of about 0.6 g/(L day). In solid state media productivities of ~1.3 g GLA/(kg substrate day) were reported. High GLA concentrations were obtained in a two stage process, whereby harvested mycelium was disrupted and incubated under defined conditions at 5 °C for 15 days. SCO, rich in GLA (15%, w/w), was the first SCO to be produced commercially from *Mucor circinelloides* by JE Sturge, UK. This fungus is used in oriental food fermentations so, not surprisingly, the oil was approved for human consumption. However, prices of the latter plant oils remained stable or even decreased, such that the SCO-

Table 8
Overview of process investigations related to microbial production of gamma-linolenic acid (GLA)

Strategy	Organism	Culture time (h)	Biomass (g/L)	GLA		Productivity		Reference
				g/L	%	Biomass	Oil	
Development of a low temperature resistance high GLA producing mutant Study of optimum conditions for GLA production	<i>Mortierella ramannia</i> (MM15-1)	216	62	5.18	18.1	0.58		[134]
							Mutant developed from parent strain IFO8187 Optimum [glucose] 300 g/L Pellets accumulated higher GLA than filamentous With optimum inoculum/agitation speed to produce 0.15–0.4 mm pellets GLA, lipid increased to 18.1%	
Optimization of GLA production in hexadecanol media	<i>Mortierella isabellina</i>	24 (+15 days)		27.1		0.054 g/day		[135]
Investigation of low temperature incubation of disrupted mycelia	<i>Mortierella isabellina</i>			22.4		0.224 g/g cells/day		[136]
Screening 48 Mucorales for highest GLA production	<i>Mucor circinelloides</i>					0.115 g/(L day)		[137]
	<i>Mucor mucedo</i>			0.38			8 strains produce >200 mg/L GLA Sunflower oil	[138]
Studies in solid state culture	<i>Cunninghamella echinulata</i> <i>Cunninghamella elegans</i> CCF1318	11 days		0.37	15.6		Medium: barley, spent malt grains, peanut oil, 21 °C 14.2 g GLA/kg substrate	[139]

GLA preparation became uncompetitive. Perhaps the market was also too small and specialized for a microbial product.

6. Microbial production of ARA

6.1. Production systems

Production of ARA in photoautotrophic algae, such as *Porphyridium cruentum* and *Parietochloris incise*, appeared to be optimal under conditions of slow growth in nitrogen-free or nitrogen-starved conditions. Slow growth rates are undesirable from a commercial productivity perspective. Kyle [47] described a method for production of ARA by the heterotrophic alga *Pythium insidiosum*. Relatively high rates of biomass growth were achieved in glucose-yeast extract medium, reaching 15 g/L in 50 h. Biomass oil content was 5–6% (0.75–0.9 g/L, of which 30–35% was ARA) but ARA yields and productivities were only 0.3 g/(L h) and 0.15 g/(L h), respectively.

Mortierella species produce ARA (and EPA) in the form of TGs. Zhu et al. [48] described a method for ARA production by *M. alpina* on glucose/defatted soybean meal and sodium nitrate with yields of 1.87 g/L ARA (17.3% of total lipids) from 31.2 g biomass in 7 days. Lan et al. [49] showed that glutamate increased ARA production, while depressing other PUFAs. In 7 days, yields of biomass and ARA were 25 g/L and 1.4 g/L, respectively. Eroshin et al. [50] showed growth-coupled lipid synthesis in *M. alpina*. Lipid synthesis rate doubled as specific growth rate was increased from 0.03 to 0.05/h, with ARA productivity of around 20 mg/(L h). Under batch conditions (189 h), ARA reached 60.4% of lipid, 18.9% of biomass and 4.5 g/L, constituting an average productivity of about 25 mg/(L h). Koike et al. [51] concluded the C:N ratio was important to achieve optimal ARA production by *M. alpina* CBS 745.68. Pellet morphology was not affected when the C/N ratio was <20, but pellet sizes increased in proportion to C/N ratio increases above 20 mg/L. Biomass and ARA concentrations were in the range 30–40 g/L and 2–3.5 g/L, respectively, after a 10-day incubation at 28°C.

Singh and Ward [52] demonstrated highest production of ARA by *M. alpina* ATCC 32222 in media containing soy flour, vegetable oils and NaNO₃ in glucose fed-batch cultures. At 25 °C, biomass and ARA yields were 52.4 g/L and 9.1 g/L, respectively, after 8 days. At 15 °C a similar biomass concentration was observed but ARA yield increased to 11.1 g/L after 11-day incubation. Kyle [53] used a medium containing glucose and soy flour/yeast extract for production of ARA by *M. alpina* in fermenters. pH was maintained at 5.5 with NaOH during the exponential growth stage. Thereafter the pH was allowed rise to 6.8 and was maintained at 6.8–7.3 by acid addition. Temperature was maintained at 28 °C and oxygen concentration at >40% saturation. After 144 h, biomass concentration was 34.5 g/L and contained 36% (w/w) (13 g/L) oil, consisting of 32.9%

ARA. The oil produced was essentially free of EPA and consequently was considered suitable for use in infant formula. Barclay [54] described a process for production of ARA by *Mortierella schmuckeri* in a medium containing glucose and soy flour or inactive bakers yeast. After 65.5 h, biomass yields were 20–22 g/L and ARA yield and productivities were 2.3 g/L and 0.84 g/(L day), respectively. Researchers have observed that many *Mortierella* strains tend to grow as pellets and considered this a disadvantage to growth rate and ARA productivity [55,56]. The strain and conditions used with *M. schmuckeri* resulted in filamentous growth which was thought to have contributed to its high productivity.

Barclay [57] described two thraustochitrid species (Strains 43B and 46B) which produced 18.5–18.6% and 38.1–39% of TFAs as ARA and total omega-6 FAs which may represent interesting sources of omega-6 fatty acids for use in eicosanoid synthetic feedstocks. Assuming culture development research with these strains can achieve 50% of the biomass productivities observed for DHA production in *Schizochytrium*, i.e. 100 g/L in 4 days, while retaining the above proportion of ARA in biomass (18.6%), these strains also offer the potential to achieve ARA productivities of >4–5 g/(L day). The high ARA-producing thraustochytrids produce an array of long chain PUFAs, including the two dominant omega-3s (DHA and EPA) and two dominant omega-6s including ARA, which may well be suited to producing the mix of PUFAs similar to human mother's milk. Use of these strains as a source of ARAs would likely require substantial strain/culture manipulation to eliminate the non-ARA PUFAs which would make ARA purification complicated and costly.

6.2. Conclusion

In conclusion, phototrophic and heterotrophic algae exhibited relatively low percentages of ARA (~2%, w/w) in biomass. While biomass concentrations of 15–30 g/L have been reported ARA productivities were typically low (0.1–0.2 g/(L day)). It has been demonstrated that *Mortierella* species, especially, *M. alpina*, can grow to biomass densities of ~50 g/L and produce more than 10 g/L ARA with productivities of 1–1.2 g/(L day). We believe that with further strain selection, combined with a fermentation development program, ARA productivities in *Mortierella* species can be raised to >5 g/(L day). *M. alpina* is a particularly attractive source for production of ARA because ARA tends to be the predominant long chain PUFA with only traces of EPA and no DHA being produced [58]. Thus, high-purity-ARA can easily be recovered by separation of the saturated and monounsaturated fatty acids. Recently isolated *Schizochytrium* strains, with the capacities to grow at high growth rates and to high densities, provide opportunities to further increase ARA productivities. Their utility for production of ARA in mixtures of PUFAs is obvious. Their potential as sources of more purified ARA

Table 9
Overview of process investigations related to microbial production of arachidonic acid (ARA)

Strategy	Organism	Culture time (h)	Biomass (g/L) culture	ARA		Productivity g/(L day)	Reference
				g/L culture	%		
					Biomass	Oil	
Study of ARA photoautrophic algal ARA production	<i>Porphyridium cruentum</i>		29		1.9		-Maximum under slow growth conditions in stationary under nitrogen starvation [24]
Study of ARA production in a recently discovered green alga	<i>Parietochloris incise</i>	38 days		2.67			Conditions: last 17 days in nitrogen free media; 90% of ARA as TG [140]
Production of ARA in an 80-L industrial fermenter	<i>Pithium insidiosum</i> ATCC 28251 <i>Mortierella schmuckeri</i>	50 65.5	15 20–22	0.3 2.3		30–35	Conditions: 24 g/L glucose, 4.8 g/L YE, 125 rpm, 1–3 SCFM [47]
Study on effect of media composition and temperature on ARA production	<i>M. alpina</i> 25 °C ATCC 32222	192	52.4	9.1		1.14	Medium: soy flour, vegetable oil, NaNO ₃ in glucose fed batch cultures [54]
Development of medium and conditions for ARA production	15 °C <i>Mortierella alpina</i>	264 144		11.1 4.3		1.01 0.72	Medium: glucose, soy flour, YE, pH maintained at 5.5 during log phase, maintained at 6.8–7.3 by acid addition, Temp 28 °C; O ₂ >40% saturation [52]
Study of effect of growth rate on lipid and ARA synthesis	<i>Mortierella alpina</i>	189		4.5	18.9	60.4	Lipid synthesis rate doubled as specific growth rate was increased from 0.03 to 0.05/h [50]
Effect of C:N ratio on pellet morphology & ARA production	<i>Mortierella alpina</i> CBS 745.68	240	30–40				C:N > 20: pellet size increased in proportion to C:N ratio, C:N < 20: no effect on morphology [51]
151 strains screened for long chain PUFA	Thraustochytrids Strains 43B, 46B					18.6	[57]

Table 10
Overview of process investigations on microbial production of docosahexaenoic acid (DHA)

Investigation/strategy	Organism	Culture time (h)	Biomass (g/L)	DHA g/L	Productivity g/(L day)			Comments	Reference
					%	Biomass Oil			
Optimize culture conditions for DHA production	<i>C. cohnii</i>	72–120	20–40	2.0	20–30	~35	0.5	N-deficient glucose medium, 28 °C, pH 7–7.8	[47]
Studies in glucose/YE/sea salt media	<i>C. cohnii</i>	91		1.6		43.6	0.52	Produced high DHA-containing lipid	[62]
C-fed batch strategies to increase biomass/DHA	<i>C. cohnii</i> , acetate, glucose, ethanol	400	109	19.0			1.15	High biomass density/DHA with acetate or ethanol	[63]
		400					0.34		
		220		11.7		33	1.27		
<i>Thraustochytrium</i> as a potential DHA producer, culture development	<i>T. aureum</i> ATCC34304	144	4.9	0.51	10.4	57	0.09	Initial identification of <i>Thraustochytrids</i> as potential producers	[66,67]
Studies on batch and fed-batch approaches to increase cell density and DHA	<i>T. roseum</i> ATCC28210	B	10.0	0.10	10.2	53	0.21	Improved biomass and DHA production	[141,142]
		FB	17.1	0.12	11.7	48	0.17		
Studies on optimization of DHA production	<i>S. limacinum</i> SR21	120		>4			>0.8	Increased yields due to strain tolerance to high C	[65]
Screened large collection (57) of strains for DHA production	<i>Thraustochytrids</i>	107	14	2.17	78	25	0.48	DHA production stimulated at high C:N, >1% glucose inhibits <i>T. aureum</i> growth	[69]
Screened <i>Thraustochytrids</i> strains from subtropical mangroves	<i>S. mangrovei</i>	52					1.27	Identified high producing strain	[70]
Investigation of non-chloride sodium forms on strain growth	<i>Thraustochytrium</i> , <i>Schizochytrium</i> species						1.08	Sodium sulphate reduced cell aggregate size	[53]
Screening of 151 newly isolated strains								Isolated high growth rate strains Characterized very high DHA producers	
To develop conditions very high DHA levels through high density culture development	<i>Schizochytrium</i> strains	90–100	200	40–45	20–22	40	~10.00	High biomass/DHA production by controlling O ₂ and glucose at <7 g/L; pH and N-feed by NH ₄ OH	[71]

preparations will be dependent on the effectiveness of measures to suppress production of non-ARA PUFAs.

A summary of key investigations related to microbial production of ARA is presented in Table 9.

7. Microbial production of DHA

A variety of microbial species have been evaluated as producers of DHA [59]. The two strains which are used commercially are the heterotrophic dinoflagellate *C. cohnii* and strains from the traustochytrid marine protists. A summary of key investigations related to microbial production of DHA is presented in Table 10.

7.1. DHA production by *Cryptocodinium cohnii*

Marine microorganisms, such as species of dinoflagellates, both photosynthetic and heterotrophic, have been characterized as a primary producer of DHA and contribute significant amounts of DNA into the marine food chain [60]. They are generally slow growing and shear sensitive, such that culturing vessel agitation to achieve oxygenation can retard growth [58].

Kyle [47] described a method for production of DHA by *C. cohnii*, which provides the basis for production of commercial DHASCO, which is incorporated into infant formulae and other foods. Oil production was promoted by nitrogen-deficient conditions. Temperature and pH optima were 28 °C and 7–7.8, respectively. In a 3–5-day incubation period biomass was produced in the range 20–40 g/L, which contained 20–30% (w/w) oil. DHA content in the oil amounted to 35% (90% of the oil was in triglyceride form). In an example fermentation, 175 g crude oil was recovered from 30 L of culture, representing a DHA yield of approximately 2 g/L. The amount of glucose used in the fermentation was 2.4 kg. Medium salinity is an important parameter influencing growth and DHA production by *C. cohnii*. Jiang and Chen [61] found the highest content (%TFA) and yield of DHA produced by strain *C. cohnii* ATCC 30556 at 9 g/L NaCl.

deSwaaf et al. [62] demonstrated production of 1.6 g/L DHA in 91 h by *C. cohnii* in glucose/yeast extract/sea-salt medium, amounting to 43.6% (w/w) of total lipid. Lipid content of biomass was 13.5%. Recently deSwaaf et al. [63] described a high density, fed-batch culture system, for DHA production by *C. cohnii*, which produced final biomass concentrations of 109 g/L, 61 g lipid/L and 19 g/L DHA in a 400-h fermentation. This was claimed to be the highest biomass, lipid and DHA yield reported in heterotrophic algae. An acetate feed produced a higher volumetric DHA productivity than a glucose feed, amounting to 48 mg/(L h) as compared to 14 mg/(L h). In a fed batch culture using ethanol 11.7 g/L DHA was produced in a total of 35 g/L lipid in 83 g/L biomass in 220 h, with volumetric DHA productivity of 53 mg/(L h).

7.2. DHA production by *Traustochytrium*/*Schizochytrium*

The best microbial sources of DHA are strains from the genus *Traustochytrium* and *Schizochytrium* (marine protists). Generally the DHA-producing traustochytrids are unicellular algal or algal-like protists, members of the order Traustochytriales; family Traustochytriaceae; genus *Traustochytrium* or *Schizochytrium*. *Schizochytrium* replicates by successive bipartition and by release of zoospores from sporangia, whereas *Traustochytrium* strains only replicate by formation of sporangia/zoospores. Individual *Traustochytrium* cells are larger and cell aggregation and clumping is often a problem [57].

Iida et al. [64] described conditions for production of 5.7 g/L biomass and 460 mg/L total lipid with 40% DHA by *T. aureum* ATCC 34304. Yokochi et al. [65] optimized production of DHA in *Schizochytrium limacinum* SR21. Maximum DHA yields of >4 g/L were obtained in both glucose (9%)- and glycerol (12%)-containing media (5-day cultures), with corn steep liquor and with salt at concentrations between 50 and 200% of the seawater salt content. This strain also grew at zero salinity, although at a lower growth rate (50% of maximum). TFA content increased with decreasing amount of nitrogen source, reaching >50% of biomass weight. The authors attributed part of the increased yields to the high tolerance of the strain to high concentrations of carbon source. This strain could be contrasted from *T. aureum*, which is completely inhibited by 0 and 200% seawater salinity. *Schizochytrium limacinum* exhibited poor growth on di- and poly-saccharides whereas *T. aureum*, *T. roseum* and *S. aggregatum* grew well on maltose and starch [66–68]. Growth of *T. aureum* was inhibited at glucose concentrations of >10 g/L. Bowles et al. [69] screened 57 traustochytrids from different locations for DHA production capability. DHA production was stimulated by a high C:N ratio. Maxima for biomass, lipid content of biomass, DHA content of lipid and DHA yield were 14 g/L, 78% (w/w), 25% (w/w) and 2.17 g/L, respectively, after 107 h. Fan et al. [70] described production of DHA by nine traustochytrid strains from subtropical mangroves. A strain of *S. mangrovei* produced 2.8 g/L DHA in 52 h at 25 °C on glucose yeast extract medium.

High DHA producing *Traustochytrium* and *Schizochytrium* strains could grow in media where a significant amount of sodium requirement was in non-chloride forms especially sodium sulphate [57]. Higher chloride concentrations are corrosive to stainless steel vessels. Chloride concentration could be reduced to 60–120 mg/L, while still achieving high biomass yields (50%, w/w of sugar substrate). Sodium sulphate has been found to reduce cell aggregate size in fermentation media typically to less than 50 or 100 µm, which is beneficial when the cells are used as aquaculture feed material. Restriction of oxygen content was also found to promote lipid production.

Table 11
Characteristics of the 151 isolated PUFA producing strains [53]

Number of strains	Characteristics	Comment
30	≥68% of total fatty acids as omega-3 fatty acids	More than any previously known strain
76	TFAs <10% (w/w) biomass	Undesirable as human dietary sources
35	Produced omega-6 fatty acids >25%,w/w total fatty acids	More than any previously known strain Potential as producers of feedstock for eicosanoid synthesis
15	≥94% of total omega-3 fatty acids as DHA	More than any previously known strain
18	≥28% (w/w) of total omega-3 fatty acids as EPA	More than any previously known strain

Barclay [57] compared PUFA production by 151 newly isolated strains with five strains from ATCC. The newly isolated strains were obtained by water filtration with polycarbonate filter followed by plating on a glucose/protein hydrolyzate/yeast extract vitamin, mineral salts-enriched seawater containing agar. A summary of characteristics of isolated strains is presented in Table 11. The distribution of key PUFAs, among selected isolates and some known existing strains, is presented in Table 12. *Schizochytrium* S9 produced 94% of its omega-3 FAs as DHA and strain ATCC 20892 produced almost 80% of its FAs as omega-3. Strains 43B and 46B produced 18.5–18.6% and 38.1–39% of TFAs as ARA and total omega-6 fatty acids, respectively. Levels of omega-6 fatty acids produced by prior *Thraustochytrium* and *Schizochytrium* strains were much less. Strain BRBG and BRBG1 may be interesting producers of EPA, producing 87–92% of their omega-3 FAs as EPA. A comparison of the growth rates of selected new isolates with prior known strains at 25 °C and 30 °C indicated the new isolates had substantially higher growth rates. Number of doublings per day for prior known strains ranged from 2.7 to 4.6 and from

3.7 to 5.7 at 25 °C and 30 °C, respectively. Corresponding values for the new isolates were 5.5–8.5 and 7.3–9.4, at 25 °C and 30 °C, respectively. Some of these new isolates were able to produce DHA at a rate of about 1.08 g/L per day per 40 g of sugar.

Baily et al. [71] described conditions for high productivity of DHA from high density cultures. The fermentation consisted of a biomass density-increasing first stage followed by a lipid production second stage. Oxygen concentration was typically greater than 4% and less than 3% saturation during the first and second stages, respectively. Corn syrup was added to the culture in a fed-batch manner to maintain a sugar concentration of about 7 g/L. Most of the sodium was supplied in a non-chloride form, sodium sulphate. The major components in the fermentation medium are listed in Table 13. The cultures used were *Schizochytrium* strains including ATCC 20888 and a wild-type isolate. Fermentation duration was typically 90–100 h. Biomass productivities were higher in the first half of the fermentation (4–5 g/(L h)), and later ranged from 2–3 g/(L h), with final biomass content reaching up to 200 g/L.

Table 12
Properties of among selected isolates and prior culture collection strains

Strain no.	Distribution of key PUFAs						Growth properties: no. of doublings per day	
	ARA	C22:5 ω-6	Total ω-6	EPA	DHA	Total ω-3	25 °C	30 °C
Selected isolates								
56B	16.7	18.4	35.1	8.6	22.5	31.1		
43B	18.5	20.5	39.0	8.1	22.1	30.2		
46B	18.6	19.5	38.1	9.0	21.7	30.7		
55B	16.2	17.7	33.8	6.3	18.1	24.4		
ATCC 20890	1.4	0	1.4	18.9	43.5	67.3		
ATCC 20891	4.2	9.5	13.7	7.3	51.2	59.6		
ATCC 20892	1.5	0	1.5	13.5	60.0	79.5		
<i>Schizochytrium</i> 31 ATCC 20888	5.0	15	20	6.9	47.4	55.8	8.5	9.4
<i>Schizochytrium</i> S9 ATCC20889	0.4	15.5	15.9	2.3	47.0	49.7	7.1	8.8
<i>Thraustochytrium</i> S42	3.3	23.8	27.1	13.5	38.7	52.2	6.6	8.3
<i>Thraustochytrium</i> U30	2.3	18.4	20.7	15.0	43.4	59.4	5.5	7.3
Prior known strains								
<i>T. aureum</i> ATCC 28211	2.9	9.8	12.7	7.7	54.6	62.9		
<i>S. aggregatum</i> ATCC 28209	10.7	12.6	23.4	4.3	20.6	26.4	4.6	5.0
<i>T. aureum</i> ATCC 34304	3.9	8.1	12.0	3.7	55.1	58.8	2.7	3.7
<i>T. striatum</i> ATCC 34473	1.6	11.4	12.9	6.9	17.8	24.7	4.6	5.3
<i>T. roseum</i> ATCC 28210	6.3	4.3	10.6	6.9	60.1	67.0	3.5	4.5
<i>T. aureum</i> ATCC 28211							4.2	5.7

Table 13
Culture medium constituents used for high density DHA producing cultures

Components	Quantity (g/L)	Trace metals and vitamins	Quantity (mg/L)
Sodium sulphate	12	MnCl ₂ ·4H ₂ O	3
KCl	0.5	ZnSO ₄ ·7H ₂ O	3
MgSO ₄ ·7H ₂ O	2.0	CoCl ₂ ·6H ₂ O	0.04
Hodag K-60 antifoam	0.35	Na ₂ MoO ₄ ·2H ₂ O	0.04
K ₂ SO ₄	0.65	CuSO ₄ ·5H ₂ O	2.0
KH ₂ PO ₄	1.0	NiSO ₄ ·6H ₂ O	2.0
(NH ₄) ₂ SO ₄	1.0	FeSO ₄ ·7H ₂ O	10
CaCl ₂ ·2H ₂ O	0.17	Thiamine	9.5
95DE corn syrup	2–10 ^a	Pantothenate	3.2
NH ₄ OH	28% ^b		

^a Concentration maintained at ~7 g/L by feeding.

^b Fed to counteract acid production. Base set point pH 5.5.

Total fatty acid content of biomass at the end of the fermentation was typically about 40% (w/w). DHA productivity averaged around 0.5 g/(L h), and reached values of 40–45 g/L of fermentation capacity. Typical DHA content of biomass and total fatty acids was 20 and 50% (w/w), respectively. As in many microbial fermentation systems, when ammonium salts are used as a source of nitrogen in combination with carbohydrates, a gradual downward pH drift is observed. Use of ammonium hydroxide served a dual purpose of feeding nitrogen for biomass development and pH control.

7.3. Conclusion

In conclusion, development of commercial processes for production of DHA has benefited from the fact that a number of organisms can accumulate high oil contents in biomass (~10–50%, w/w) and produce a high percentage of total lipids as DHA (30–70%). High biomass densities (up to 109 g/L) and DHA concentrations of ~20 g/L have been achieved in carbon fed batch cultures of marine species, such as the dinoflagellates, *C. cohnii*, although prolonged culture times (400 h) were required. These studies have demonstrated that DHA productivities of 1–1.5 g/(L day) are achievable with this strain. Studies with thraustochytrids have established these marine protists as preeminent industrial strains for production of DHA. Initial research, at relatively, low cell densities, (5–20 g/L) established the capacities of *Thraustochytrium* species to accumulate greater than 50% of their lipids as DHA and to produce >1 g DHA/L of culture, with productivities of about 0.2 g/(L day). Related *Schizochytrium* species with higher growth rates were isolated. Under glucose and nitrogen-fed batch conditions, with incorporation of sodium sulphate as main sodium source and with control of glucose concentration, pH and O₂, selected strains could grow to high biomass densities (200 g/L) in short fermentation cycles (90–100 h), accumulating 40–45 g/L DHA and hence DHA productivities of ~10 g/(L day).

8. Microbial production of EPA

8.1. EPA production systems

A summary of key investigations related to microbial production of EPA is presented in Table 14. Many authors have investigated processes for production of EPA by autotrophic algae, such as *P. tricornutum* and *Monodus subterraneus*, and the complex nature of lipid species in these kinds of organisms, has been characterized [72–77]. Much consideration has also been given to the design of photobioreactors, including horizontal tube systems. Nevertheless EPA productivities have remained low (<0.1 g/(L day)) in commercial terms. Continuous culture, with the heterotrophic diatom *Nitzschia laevis*, resulted in highest EPA productivities of 73 mg/(L day) using a glucose feed [78]. In a high cell density system maximum cell dry weight and EPA yields were 22.1 g/L and 695 mg/L, respectively, in a 14-day incubation [79].

Various strategies have also been implemented to increase EPA productivities in heterotrophic systems, for example, with *Nitzschia alba* and *N. laevis* in glucose fed-batch systems, use nitrogen-depletion systems which induce lipid production, control of glucose-silicate ratios and use of cell recycle approaches to reduce fermentation product toxicity, thereby achieving productivities of 0.3 g/(L day). Kyle and Gladue [80] described a method for production of EPA, by the heterotrophic diatom *Nitzschia alba*, in a medium containing nitrogen and silicate with a glucose feed, where the ratio of glucose to silicate was controlled. Initial cell doubling time was 4–8 h and the later oleaginous stage was induced by depletion of nitrogen. Biomass concentration increased to 40–50 g/L in a fermentation of about 100 h. Approximately 40–50% of biomass (16–25 g/L) was oil, 3–5% of which (0.5–1.25 g/L) was EPA. Wen and Chen [78] controlled glucose feed and used perfusion systems to reduce toxicity and minimize glucose content in the reactor effluent.

A variety of strategies were also used to enhance production of EPA in a *M. alpina* strain, including use of lower incubation temperatures, addition of linseed oil substrate, and development of Δ -12 desaturase deficient mutants. During an isolation and screening of thraustochytrids for high DHA-producing, two *Schizochytrium* strains were found which produced 87–92% of their omega-3 fatty acids as EPA.

8.2. Conclusions

In conclusion, photoautotrophic algae, such as *P. tricornutum* and *Monodus*, generally have potential to grow to high cell densities of about 10 g/L, with EPA content of biomass of around 2–3%. Growth rates are regulated in part by light availability to the culture and this keeps EPA productivities below 0.1 g/(L day). While EPA contents of heterotrophic algae, such as *Nitzschia laevis* were similar to

Table 14
Overview of process investigations related to microbial production of EPA

Strategy	Organism	Culture time (h)	Biomass (g/L)	EPA			Productivity (g/(L day))	Comments	Reference
				g/L	%	Biomass			
Optimization of EPA production in batch culture; effect of reactor surface to volume ratio and dilution rate on growth and EPA productivity	<i>P. tricornutum</i>		4–6	0.08–0.13	~3.3	28–35	0.025	The smaller the culture unit the higher the biomass growth rate	[72–74]
Effect of dilution rate on EPA production in 50 L tubular photobioreactor	<i>Thraustochytrid strains</i>							Biomass and EPA productivities were higher at a 0.3/day dilution rate than at 0.15/day	
Engineering evaluation of photobioreactor EPA production process	<i>P. tricornutum</i>						0.047	Maximum productivity at dilution rate of 0.36/day	[143]
							0.04	2 g/day biomass, assumed containing 2% EPA	[32]
Characterization and optimization of EPA production parameters	<i>N. alba</i> ATCC 40775	100	40–50	0.5–1.25	1–3	3–5	0.1–0.3	Horizontal tubular photobioreactor	[26]
Development of continuous culture methods	<i>N. laevis</i>						0.073	Glucose fed batch conditions, control of glucose/silicate ratio, initial cell doubling times 4–8 h	[78]
Development of high cell density system	<i>N. laevis</i>	336	22.1	0.70			0.035	Glucose fed system produced high EPA productivities	[79]
Development of 'perfusion' system for glucose feeding and toxic product removal	<i>N. laevis</i>			1.11			Low	Feed [glucose] 50 g/L	[79]
To increase EPA productivity in perfusion system and reduce effluent glucose	<i>N. laevis</i>						0.175	Design involved cell recycle High glucose in effluent	[144]
Manipulation of cell density in outdoor cultures	<i>M. subterraneus</i>							Feed [glucose] 15 g/L	
Studies on production of EPA and characterization of culture conditions	<i>M. alpina</i> 1S-4	240		0.30	2.7		0.059	Low glucose in effluent	[145]
Studies at low temperature with linseed oil feed	<i>M. alpina</i>	240	43	0.6			0.06	High EPA productivity achieved EPA produced at low temps which activates EPA producing enzymes	[146]
Studies on production of EPA from alpha linolenic by a Δ-12 desaturase deficient mutant	Δ-12 desaturase defective mutant of <i>M. alpina</i> 1S-4	240		1.0	6.4	20	0.1	Incubation 11 °C, increasing linseed oil to 4%, biomass/EPA yield increased	[147–149]
ARA producing strains from screening of 151 Thraustochytrids for long chain PUFAs	Thraustochytrid strains							Incubation 11 °C, increasing linseed oil to 4%, biomass/EPA yield increased	
								Conditions: 20 °C, 1% YE, 3% linseed oil, 1% glucose; 90% EPA as TGs.	[150]
								Main lipids: EPA:AA:palmitic:oleic:linoleic: lignoceric = 20:8:5:20:10:4	[57]

photoautotrophic algae (1–3%), the non dependence on light facilitates development of higher cell densities (50 g/L) producing increased productivities of 0.1–0.3 g/(L day). Marine fungi, such as *M. alpina*, grew at similar cell densities to *N. laeva*, and exhibited similar EPA productivities. A Δ -12 desaturase defective mutant of *Mortierella* produced somewhat higher EPA content in biomass (6.4%, w/w). *Schizochytrium* species, producing high proportions of EPA in their lipids, have significant potential as commercial EPA producers. Based on culture development studies with *Schizochytrium*, for DHA production, we have assumed it would be possible to develop EPA production processes with these two strains achieving cell densities of 100 g/L in 100 h containing 40% (w/w) lipids. Assuming omega fatty acids accounted for 35% of TFAs, EPA productivities of 2.5–3 g/(L day) should be possible.

9. Characteristics of PUFA producers

Generally required characteristics of industrial SCO-producing microorganisms are no different to the properties required for microbial producers of other industrial products and single cell proteins [11]. The organisms must be genetically stable, so that they do not loose the desirable oil-producing characteristics over time or assume undesirable process- or product-related characteristics, such that the fermentation process remains consistent from batch to batch. This stability is important from a regulatory perspective as agencies, such as the FDA, require that the organism, and hence the process, does not deviate significantly from the process which received regulatory approval. The strains must be non-pathogenic and non-toxin-forming, so that they are both safe to work with for employees in the production plant and that the product is safe for incorporation into feeds, foods, as dietary supplements and as pharmaceuticals. For aerobic fermentations (includes single cell oil production processes) the organism has to be able to withstand shear due to impeller mixing and aeration. The organism should be capable of high growth rates and exhibit high rates of product formation. Where the product is an intermediary metabolite, the strain should not further metabolize or transform the desired product, that is, rates of biosynthesis upstream with respect to the product should be high and rates of catabolism of the product should be negligible. The organism should be capable of using low-cost fermentation media and, for bulk products, should exhibit high conversion yields of product from substrate. There should be potential to manipulate cells and fermentation conditions, to maximize desired product yield and minimize formation of undesired by-product. The organism and culture conditions should be such as to facilitate cost effective recovery of the product to desired specifications.

Some of the key specific characteristics of SCO producers are listed in Table 15. While most PUFA-

Table 15

Characteristics of a good single cell industrial oil producer

Oil specific properties

- Capable of accumulating a high percentage of lipid
- Capable of heterotrophic growth
- Capable of growth at low salinities
- Capable of growth/product formation at higher temperatures (>30 °C)
- Produces a high proportion of total lipid as the desired product
- Produces a low content of PUFAs not desired in the product
- The organism should have non-pigmented, white or colorless cells
- PUFA should be present as triglyceride if for human consumption
- For mariculture the organism aggregate size should be small enough to be consumed by the small fish feeders
- The oil should be easily extracted from the biomass

producing strains have been isolated from marine or salt-containing environments, they should be capable of growth at low salinities and at higher temperatures, such that high productivities are achievable. The organism should not be heavily pigmented, given the pigment would likely be present in the oil. The oil should contain high concentrations of the desired product and preferably contain low levels of other PUFAs, which are not easily separated from the desired product. The desired PUFA should be in an esterified form appropriate for the application (i.e. in glyceride form for human consumption) and, for mariculture applications,

Table 16

Summary of key characteristics of SCO production by *Schizochytrium*

Strain description [151]

- A species from the thraustochytrids, obligate marine fungoid protists, with a specific requirement for sodium ions intensive screening produced a large number of strains with diverse PUFA production capability (isolation approach)
- Very high percentage of total fatty acids as PUFAs

DHA

- Very high percentage of oil in biomass
- Glucose concentration >1% inhibits growth, importance of feeding and controlling glucose concentration
- pH controlled in range optimizes growth rate
- O₂ controlled at 40% saturation to maintain high growth rate
- Nitrogen deficient conditions favour DHA production
- Feeding of N as NH₄OH during pH control, maintains N-deficient conditions
- Use of sodium sulphate as sodium source reduced negative harsh effect of Cl on equipment
- Sodium sulphate reduces biomass aggregate size as required for mariculture applications
- Achievement of very high density cultures (200 g/L) in 4 days
- Achievement of very high DHA yields and productivities

ARA and EPA

- Potential to select and manipulate strains and culture conditions to achieve high productivities of ARA or EPA, while minimizing concentrations of other PUFAs for ease of product recovery
- Potential to produce desired combination of ARA and DHA in a single strain for use in milk and baby food formulae. However, currently not used for that application. The presence of EPA precludes use as breast-milk substitutes

the harvested oil-containing microbes should be small enough for small fish/larval consumption.

Some of the key characteristics of processes for single cell oil production by *Schizochytrium* species are presented in Table 16. *Schizochytrium* strains have been shown to exhibit diverse PUFA-production capabilities, with PUFAs in the form of triglycerides and with very high percentages of lipids in the form of PUFAs. Processes for DHA production from high DHA-containing strains have high rates of growth and product formation by control of glucose, nitrogen, sodium and oxygen concentrations as well as temperature and pH, thereby achieving very high cell densities and DHA productivities. There is potential to produce *Schizochytrium*-based oil, low in EPA, for the infant formula market. With additional strain selection, manipulation and culture development there is potential to develop *Schizochytrium*-based fermentations for production of ARA and EPA.

C. cohnii was the first microbial strain used for commercial production of DHA for infant formula because of its low content of EPA. High biomass densities and commercially acceptable DHA productivities are achieved with this dinoflagellate in media where carbon concentration, pH and temperature are controlled under nitrogen-deficient conditions. Acetate or ethanol may be used as alternative substrates to glucose with potential to achieve higher DHA productivities, although their higher substrate costs and material handling challenges will reduce some of the productivity advantage. A summary of the key characteristics of *C. cohnii* SCO production processes is presented in Table 17.

Mortierella species have been found to be excellent producers of ARA, producing up to 60% of total fatty acids as ARA. This strain is currently regarded as the most effective industrial producer of ARA. Optimized processes with high ARA-producing *M. alpina* species involve use of complex nitrogen sources, glucose fed-batch cultures, temperature control and possibly separate pH control regimes for growth (pH 5.5) and ARA production (pH 7.0). Selected *Mortierella* strains may be used for industrial production of GLA. Low temperatures promote GLA production and a two stage process with a higher growth temperature followed by low temperature mycelial storage has been reported. The morphology type has also been reported to influence GLA production. At lower temperatures some *Mortierella* strains produce substantial amounts of EPA and use linseed oil as a precursor substrate for EPA production. The lower productivity associated with the required low culture temperature is a disadvantage from a commercial perspective. Improved EPA production has been reported from a Δ -12 desaturase defective mutant. As will be seen in Section 10, Δ -5 and combined Δ -5, Δ -12 defective Δ have been shown to produce high concentrations of dihomo-GLA and 8,11,14,17-*cis*-eicosatetraenoic acid (20:4, n3), respectively. Table 18 summarizes the key characteristics of *Mortierella*-SCO production processes.

Table 17

Summary of key characteristics of SCO production by *Cryptocodinium cohnii*

High percentage of total fatty acids as PUFAs
Form of DHA
Production supported by N-deficient conditions
Optimum temperature around 28 °C
Optimum pH 7–7.8
High biomass produced with carbon fed-batch strategies
Higher DHA productivities achieved with acetate or ethanol feeds in place of glucose

10. Biochemical pathways for production of PUFAs

PUFAs are generally synthesized by modification of saturated fatty acid precursors, whereby desaturase enzymes insert double bonds at specific carbon locations in the fatty acid and a fatty acid elongation system extends the chain in two-carbon increments [81–83]. Synthesis of arachidonic acid (20:4n6) and EPA (20:5n3) in humans predominantly starts with plant precursors, linoleic acid (18:2n6) and linolenic acid (18:3n3), respectively, and involves alternating fatty acid desaturation and elongation reactions, mediated by specific desaturase and elongase enzymes. Production of DHA (22:6n3) involves a double elongation of 20:5n3 to 22:5n3 and then to 24:5n3 followed by its Δ -6 desaturation to 24:6n3 and one beta-oxidation cycle (in peroxisomes) to produce 22:6n3. A novel PUFA biosyn-

Table 18

Summary of key characteristics of SCO production by *Mortierella* species

ARA
Production of large proportion of ARA in oil by <i>M. alpina</i> , up to 60%
Relatively high biomass yields achieved through pH control at 5.5
Higher pH after growth (~7) favors ARA production
High biomass achieved in glucose fed-batch cultures
Complex nitrogen source promotes good growth
<i>Mortierella alpina</i> appears to be a good robust strain for industrial production
There is potential to increase biomass growth rate and ARA productivity by further fermentation development studies, with greater process control and perhaps by screening for strains with faster doubling times
EPA
Lower temperatures favour EPA production, a disadvantage from a growth rate perspective
Linseed oil addition promotes EPA production
Improved EPA production achieved in Δ -12 desaturase defective mutant
GLA
Two stage fermentation, with growth at 23 °C and storage of recovered mycelium at 5 °C promoted GLA accumulation
GLA accumulation has been achieved in recovered disrupted mycelia
A low temperature resistant mutant accumulated GLA at 26 °C
Optimum pH controlled in the latter at 4.0
Higher GLA production in pellets rather than filamentous mycelia
Pellet size also affected GLA content, optimum at 0.15–0.4 mm.
Optimum pellet size controlled by control of inoculum size and agitation speed

thetic pathway has recently been characterized in some prokaryotic and eukaryotic organisms that does not depend on the fatty acid desaturase/elongase system but rather exploits a polyketide synthase mechanism to produce 20:5n3 and 22:6n3 [84]. Strategies for development of new systems for production of PUFAs are beginning to benefit from biochemical and genetic studies on the key biosynthesis enzymes involved namely the desaturases, elongases and polyketide synthases. Hence, the following sections will describe some of the pertinent investigations.

10.1. Desaturation and elongation processes

The hydrophobic/membrane-bound nature of FA desaturases, which consist of at least three functions (Cyt b5 reductase, Cyt b5 and the terminal reductase) and the lack of availability or high cost of the substrates (acylCoA or acyl-CoA/phospholipids complexes, preferably ^{14}C -labelled for assay purposes) have retarded research progress on biochemical mechanisms of desaturases. Studies using mutants lacking a desaturase (other than Δ -9) were frustrated by the inability to differentiate mutated phenotypes from the non-mutated wild type. That notwithstanding, studies have been completed on mutants deficient in Δ -9, Δ -12, Δ -6 and Δ -5 desaturases [85]. While different desaturases in a single species and desaturases in different species lack overall similarity, certain structural and functional features, most notably three histidine-rich motifs, appear to be highly conserved among all desaturases [86,87]. While different desaturase genes exhibit a general lack of sequence homology, the structural similarities have been demonstrated especially by heterologous expression of microbial desaturases in other microbial species, as well as in plants and animals [88–90]. This heterologous functionality represents the key to being able to transfer microbial desaturase genes to plants with the potential to modify and enrich seed oil crops with desired PUFAs [91].

The Δ -9 desaturase, which catalyses insertion of the first double bond into the fatty acid (C9–C10), preferentially uses palmitate (16:0) but can also use stearate (18:0) as substrate [92,93]. It uses acylCoA as substrate in contrast to other desaturases, which use phospholipids bound acyl groups. The fungal enzyme is membrane-bound associated with the ER. The Δ -9 desaturases are the most conserved of all the desaturases and mutants, deficient in the enzyme, are unable to grow in media lacking exogenous unsaturated fatty acids [94]. Removal of the activities of other desaturases by mutation typically does not prevent growth. *M. alpina* appears to contain up to three genes for Δ -9 desaturase, one of which is expressed in all *M. alpina* strains, while expression of a second encoding a similar protein varies among strains. A third desaturase gene expresses an enzyme having quite a different composition and substrate specificity, in that it only uses stearic acid as substrate [95]. Δ -9 Gene expression appears to be repressed by Δ -9-desaturated FAs in the medium [96,97] and by cultivation temperature [98].

The Δ -12 desaturases, which convert oleic (18:1n-9) to linoleic (18:2n-6), have been characterized from a variety of microbial species, and the genes from *M. alpina* and some other species, which has been cloned, are relatively short encoding proteins having 350–400 amino acids, as compared with Δ -9 desaturase genes, presumably because the Δ -12 desaturase system does not have a cytochrome b5 domain [93].

The Δ -6 desaturases convert linoleic (18:2n-6) to GLA (18:3n-3) [n-6 pathway] and GLA(18:3n-3) to 18:4n-3 [n-3 pathway]. The gene from *M. alpina*, which has been cloned, encodes a polypeptide of 457 amino acids and contains a cyt b5 domain [98,99]. The Δ -5 desaturase can convert di-homo-GLA(20:3n-6) to ARA (20:4n-6) [n-6 pathway] or 20:4n-3 to EPA (20:5n-3) [n-3 pathway]. The gene from *M. alpina* has been cloned and encodes a polypeptide of 446 amino acids [89]. The Δ -6 and Δ -5 desaturases have similar sequence homology, introduce double bonds into the fatty acid to the carboxyl end relative to the initial Δ -9 site and share common features differentiating them from enzymes that desaturate fatty acids in the methyl end of the Δ -9 site.

A Δ -4 desaturase could catalyze the conversion of docosapentaenoic acid (22n-3) to form DHA (22:6n-3), and a gene encoding a protein with Δ -4 desaturase activity in yeast and plants has been cloned from *Thraustochytrium* sp. [91]. In contrast, in the related *Schizochytrium* species, EPA biosynthesis is not affected by anaerobic growth suggesting a fatty acid oxygen-requiring desaturation route to EPA was not necessary [100].

Elongases are multiunit membrane-associated proteins and like desaturases are difficult to investigate, such that their mechanisms remain obscure. The reaction sequence for FA elongation is the same as that for FA synthesis, involving four linked steps: condensation of the acyl group to a malonylCoA producing a ketoacyl product and CO_2 , reduction to the γ -hydroxyl moiety, its dehydration to an enoyl group, followed by a second reduction, to give the elongated FA. The key difference is the membrane bound nature of the elongase, compared to the cytosolic nature of de novo fatty acid synthesis, and the specificity and rate limiting properties of the first enzyme.

A gene encoding a discrete elongase for converting 16:0 to 18:0 was identified in *M. alpina* [101]. A second gene, encoding a 318 amino acid polypeptide with elongase activity specific for 18:0, was later isolated, that exhibited activity not only to the 18:3n-6 natural substrate for the *Mortierella* n-6 pathway, but also for the 18:4n-3 substrate of the n-3 pathway, which *M. alpina* does not possess. Extension of C18 to C20 and C20 to C22 appear to be carried out by separate specific elongases. It has been suggested that the unique part of the elongase system is the condensing enzyme and that the other three components may be supplied by the existing fatty acid complex.

In plants and animals, the fatty acid elongation system is generally thought to involve a rate-limiting condensing enzyme together with two reductases and a dehydrase, with

the latter three being present constitutively or induced by the condensing enzyme [102,103]. The condensing enzyme is thought to determine substrate specificity in terms of chain length and degree of unsaturation. The GL elongase 1 from *M. alpina* acts specifically on two products of Δ -6 desaturation, 18:3 n-6, Δ -6,9,12 and 18:4 n-3 Δ -6,9,12,15 [104]. Qiu et al. [91] provide a useful discussion on the properties of PUFA fatty acid elongases of *Isochrysis galbana* [124], *M. alpina* and other organisms.

10.2. Polyketide-like PUFA synthesis

While PUFA synthesis from acetyl CoA, as it occurs in animals and plant, needs about 30 enzymes, a much simpler pathway involving a specialized polyketide synthase system, has been characterized for PUFA production in marine prokaryotic and eukaryotic micro-organisms [84]. Synthesis of polyketide secondary metabolites involves a set of enzymatic reactions analogous to those of fatty acid synthesis [105,106]. Polyketide synthesis (PKS) may be used to make novel antibiotics [107]. Novel approaches to PUFA production involved use of a polyketide-like system, which utilize the same four basic reactions of FAS, but the cycle is often shortened to produce a carbon chain with many keto, hydroxyl and C=C double bonds. PKS proteins have sequence similarity with FAS enzymes.

Shewanella putrefaciens contains such a PKS-like gene cluster expressing the EPA synthesis pathway, containing a minimum of five open reading frames (ORFs), totaling 20 kb within the chromosomal fragment. EPA production has been achieved in *E. coli*, transformed with the latter gene cluster in a 38 kb DNA fragment [108]. The 5 *Shewanella* genes expressed in *E. coli* encode a protein complex that can synthesize EPA, without participation of *E. coli* FAS or components, such as 16:0 ACP. In *E. coli*, as in *Shewanella*, EPA accumulates in the sn-2 position of the PL fraction.

The protein sequences encoded from the 5 ORFs resulted in identification of 11 putative enzyme domains. Eight of these domains, including malonyl CoA:ACP acetyltransferase, 3-ketoacyltransferase, pantethein transferase, chain length (or chain initiation) factor and a cluster of six putative ACP domains, were more strongly related to PKS rather than FAS proteins. There were three other regions, which appeared to be homologues of the bacterial FAS proteins, enoyl reductase and dehydrase (two regions) from bacteria [109]. The psychrotolerant piezophilic deep-sea PUFA-producing bacterium, *P. profundum*, contains a 33 kbp locus which includes four of the five genes required for EPA biosynthesis, mediated by a PKS-synthesis mechanism [110]. Mutation studies indicate that a regulatory factor appears to co-ordinate both increased expression of the four genes and elevated EPA production. A similar PKS-like gene cluster is present in *V. marinus* [111].

Other PUFA-producing strains, including *Schizochytrium*, also appear to contain these systems. The membrane-bound desaturase and elongase enzymes from *Schizochy-*

trium appear not to be involved in PUFA synthesis, which is mediated by a PKS protein complex, three of the protein domains of which have high sequence similarities with those encoded by the *Shewanella* PKS gene cluster. It has been suggested that the PKS pathway predominates in *Schizochytrium* whereas a desaturation/elongation pathway may dominate in *Thraustochytrium* [91].

Since the PKS cycle would add 2-C units to the chain, while EPA and DHA double bonds are located at every third carbon, it has been suggested that this could be achieved by generation of double bonds at Δ -14 and Δ -8 of EPA by 2-*trans*, 2-*cis* isomerization, followed by incorporation of the *cis*-double bond into the elongating fatty acid by an as yet unidentified enzyme, perhaps represented by one of the unassigned PKS protein domains.

11. Production of metabolic intermediates

As in other areas of microbiology, knowledge of microbial biosynthetic pathways allows us to eliminate specific enzymes through mutation or molecular approaches and thus to accumulate high amounts of intermediary metabolites. In this manner a Δ -5 desaturase mutant of the ARA-producing *M. alpina* 1S4 strain accumulated high quantities of dihomo- γ -linolenic acid (DHGLA), amounting to 44% of total glyceride fatty acids [112]. DHGLA has potential medical applications for treatment of viral infections, specific cancers, and atopy of the skin and mucosa [113]. A Δ -5, Δ -12 desaturase mutant of the same strain accumulated large amounts of 8,11,14,17-*cis*-eicosatetraenoic acid (20:4n3), amounting to 37% of total fatty acids [114]. Details of the culture procedures used in these examples are summarized in Table 19.

12. Synthesis of PUFAs in transgenic plants

Plant fatty acid biosynthesis is carried out exclusively in the plastid by the fatty acid synthase complex [115,116]. Desaturation reactions facilitate production of (18:1n9), linoleic acid (18:2n6), γ -linolenic acid (18:3n6), α -linolenic acid (18:3n3) and octadecatetraenoic acid (18:4n3). Hence, higher plants do not synthesize long chain PUFAs. A number of separate desaturase/elongase enzymes are required for fatty acid synthesis from LA (common in plants) to long chain PUFAs. Getting plant cells to produce EPA/DHA likely requires expression of five or six introduced enzymes as well as system engineering to produce high levels and to prevent further onward metabolism of the desired products. Transforming host plants and plant cells with an expression cassette, comprising a transcriptional/translational initiation region joined to a gene or component of a PKS-like system capable of modulating the production of PUFAs, can result in alterations in the PUFA profile in the host cells. The expression cassette, with PKS-like gene activity, encodes a

Table 19
Overview of process investigations related to microbial production of metabolic intermediates

Strategy	Organism	Culture Time (h)	Biomass (g/L)	Production		Productivity		Comments	Reference
				g/L	%	Biomass	Oil (g/(L day))		
Dihomo-gamma-linolenic acid	Δ -5 desaturase defective mutant of <i>M. alpina</i> IS4 (ARA producer)	288	7			43.9	0.58	12-day cultivation in industrial fermenter at 28 °C. Other FAs (%): palmitic, 18.2; stearic, 7.9; oleic, 7.5; linoleic, 4.4; GLA, 3.2; ARA, 0.4; lignoceric, 7.7.	[112]
Develop process for metabolic intermediate production by use of enzyme deficient mutants									
8,11,14,17- <i>cis</i> -eicosatetraenoic acid 20:4n-3	Δ -5; Δ -12 desaturase defective mutant of <i>M. alpina</i> IS4 (ARA producer)	288	23	2.24	9.7	37.1	0.19	12-day culture on linseed oil.	[152]
Develop process for metabolic intermediate production by use of enzyme deficient mutants								Only other PUFA was Dihomo-GLA (4.9% TFAs)	
Lipid distribution: TG: DG:PL FFA = 82:7:9:2									

polypeptide capable of increasing the amount of one or more PUFAs in the cell. In transgenic plants, using a PKS-like system, PUFAs accumulate in the cytoplasm.

Lopez and Maroto [117] concluded that most of the basic tools for genetic engineering of oilseed plants to confer on them the ability to produce PUFAs are already developed. However, the latter authors note that micro-organisms are likely to be important competitors to plants for PUFA production and recognize that PUFA crops for production of ARA, EPA and DHA will be hard to develop [118]. It is our opinion that plants will indeed be engineered to produce PUFAs as a dietary source of these fatty acids. However, micro-organisms have the advantage of being capable of producing individual PUFAs as the dominant PUFA in the lipid material, thereby greatly simplifying the recovery and purification process. In this regard plant and microbial sources of PUFAs are likely to address different target markets.

13. Physico-chemical methods for recovery/purification of PUFAs

Typical processes for extraction of oils from plant material most commonly involve countercurrent solvent extraction from dried plant material, usually after reduction of particle size by flaking to facilitate access of the solvent to the plant structures followed by solvent removal by decanting and residual traces removed by vacuum evaporation [119,120]. Other extraction approaches can use supercritical fluid extraction. Most fish oils are produced via a wet reduction process under inert gas or in closed containers to reduce oxidation by atmospheric oxygen. Cooking partially sterilizes the oil, denatures protein and facilitates oil release, followed by mechanical decanting and pressing [12].

With microbial oil-containing biomass, the cells or mycelium biosolids are typically harvested by centrifugation or filtration to remove the majority of the aqueous material after which the cells may be dried and solvent extracted. Homogenization of an aqueous suspension to reduce biomass particle size to less than 10 microns followed by extraction with a non-water miscible organic solvent has been found to be an effective extraction method [119]. Alkali pretreatment of the microbial biomass can also facilitate solvent extraction of the single cell oil. Alternatively moisture content of the harvested cells may be reduced by spray drying or freeze drying prior to application of a solvent extraction step.

Recovered oils can be further processed if desired to concentrate the HUFA fraction by the process of winterization, whereby the temperature of the oil is reduced to effect precipitation of the more saturated lipids which may then be separated out. Oils may be further purified by standard processes through filtration, bleaching, deodorization, polishing and antioxidants may be added to prolong shelf

Table 20
Summary of general PUFA enrichment processes

Method	Procedure
Winterization	Reduce temperature to render more saturated fats insoluble Separate out insoluble fraction
Glyceride hydrolysis	Hydrolyze glycerides to glycerol and free fatty acids using saponification or lipase enzymes
Urea complexation	Add urea and aqueous ethanol, heat to solubilize the fatty acid mixture Reduce temperature to crystallize urea-saturated complexes Centrifuge/filter out crystals Acidify supernatant Extract above supernatant with hexane Recover PUFAs as FFAs from hexane by evaporation
Enzyme splitting	Promote lipase selective esterification of more saturated fatty acids Separate concentration of PUFAs in FFA fraction from esterified saturated fatty acids Repeat esterification for further PUFA enrichment
PUFA transformations	Esterify PUFA free fatty acids to produce esters (ethyl-, glyceryl-, sugar-, other) Interesterifications to enrich non-HUFA oils with PUFAs

life. The main PUFA enrichment processes are summarized in Table 20.

Physico-chemical methods for further fractionation of HUFA mixtures include urea inclusion/fractionation distillations, liquid chromatography and enzyme-based procedures. The urea fractionation methods for purification of concentrated EPA and its ethyl ester from fish oils are described by Fujita and Makuta [121] and Haagsma et al. [122]. They are complicated and likely costly procedures from a production standpoint and the need to use organic solvents is also a disadvantage. HPLC and silver ion exchange column chromatography have also been evaluated but these methods are not amenable to scale up.

14. Enzymatic methods for refining/processing of PUFAs

Applications of enzymes in bioprocessing are especially advantageous because they act under mild reaction conditions. Long chain PUFAs are highly labile and reaction methods which exploit extremes of pH or temperature can destroy the all *cis* nature of omega-3 fatty acids, such as EPA or DHA, by oxidation, *cis*–*trans* isomerization or migration of double bonds.

Lipase enzymatic reactions may be used to enrich PUFAs in oils and to produce different forms and compositions of PUFAs and PUFAs, as triglycerides, phospholipids, other fatty acid esters and free fatty acids. A possible strategy for

enriching PUFAs would be to find a commercial lipase specific for selective hydrolysis of the PUFA or for the more saturated fatty acids. Such an enzyme has not been found. Ester hydrolysis is favoured in predominantly aqueous conditions. However, since lipase reactions are reversible the enzymes can be used to promote esterification, interesterification and transesterification reactions, reactions which are favoured when the amount of water in the reaction mixture is restricted [12]. Thus, another strategy is to attempt selective esterification of FFAs and this has been more effective than the hydrolysis reaction. In theory, by judicious choice of lipase, with respect to lipase triglyceride positional specificity, ester specificity and fatty acid chain length specificity, and by varying substrate, water content and use of hydrophobic solvents and other conditions, the reaction can be tailored to produce different product forms.

Some examples of lipase transformations of PUFAs are illustrated in Table 21. PUFA-containing oils may be hydrolyzed by heating with ethanol under alkaline conditions but large scale chemical hydrolysis can isomerize the PUFAs and in addition generates large volumes of wastes. Example 1 illustrates how a non-positionally specific lipase *hydrolysis* results in a release of ~70% of both the total fatty acids and DHA as FFAs. Where the desired PUFA is concentrated at either the 2- or 1,3-positions of the glyceride, 2- or 1,3-specific lipases may be used to concentrate the PUFA and this has indeed been achieved. Much of the DHA in fish oil is esterified to the 2-position of the glycerol molecule. Example 2 illustrates how *hydrolysis* of cod liver oil (DHA content 9.64%) with a 1,3-specific lipase predominantly removes more saturated fatty acids from the 1,3 positions, thereby enriching a residual monoglyceride fraction (DHA content 29.17%).

Attempts have been made to use lipases, in both the hydrolysis and esterification directions, to selectively enrich PUFAs, based on their differential rates of transformation for PUFAs versus more saturated FAs. Such strategies were not very effective for the hydrolytic reaction. However, example 3 illustrates that when FFAs, prepared from tuna oil (DHA content 23.2%), are *esterified* with lauryl alcohol, in a reaction mediated by *Rhizopus delemar* lipase, the enzyme preferentially esterifies the more saturated fatty acids, thereby concentrating the DHA (84%) in the unconverted FFA fraction. Ethyl esters of PUFAs are of great interest to the pharmaceutical industry. If the products of a lipase hydrolytic reaction are subsequently reacted with ethanol, mediated by a lipase which will fully esterify the PUFA FFA to ethyl PUFA, the various constituents can easily be separated and an ethyl PUFA of >90% purity can be recovered [123]. A transesterification strategy can be used to concentrate PUFAs in the glyceride fraction (Example 4). By incubating cod liver oil with isopropanol in the presence of lipase under defined conditions, the lipase again preferentially transesterifies more unsaturated fatty acids from the oil triglycerides to isopropanol, thereby concentrating the PUFAs from ~19.7% to ~40% in the residual glycerides.

Table 21
Examples of applications of lipases in purification/transformation of HUFAs

Example no.	Enzyme process	PUFA substrate	Specific HUFA concentration	Reaction conditions	%Transformation	Concentration of HUFA (fraction)	Reference
1	Hydrolysis	Tuna oil	DHA 22.9%	Oil:water:lipase AK [*] 2.5 g: 2.5:2500 U, 16 h, 30 °C	68.4 (TFAs), 71.9 (DHA)		[123]
2	Hydrolysis	Cod liver oil	DHA 9.64	3.33 g oil, 1.66 mL buffer, 6000 U lipase N [*] , 72 h, 30 °C		DHA in MG 29.17%	[153]
3	Alcohol esterification	Tuna FFAs	DHA 23.2%	8 g FFAs/lauryl alcohol (1.2, mol/mol); 200 U Lipase R. delemar ^{***} , 2 g water, 20 h, 30 °C	73%	DHA 84% (FFAs)	[123]
4	Trans-esterification	Cod liver oil	EPA/DHA 19.7%	0.5 mL oil, 2 mL isopropanol, 0.1 mL phosphate buffer, 1000 U lipase CES [*] , 10 °C	~50% FFAs + isopropanol ester	DHA, EPA in glyceride fraction (~40%)	[154,155]
5	Glycerol esterification	Cod liver oil concentration PUFA FFA fraction	EPA + DHA 81.4%	0.4 g FFA, 2 g glycerol, 5% H ₂ O + molecular sieve, 5000 U lipase PS-30 [*] ; 3 mL hexane	82.5	DHA/EPA in glycerides 76–78%	[156]
6	Glycerol esterification	Cod liver oil concentration PUFA FFA fraction	EPA+DHA 81.4%	1 g 1,2 isopropylidene glycerol, 7.5 mM FFAs, 3 mL hexane, 2.5% water 6000 U Lipase IM-60 ^{**} , 12 h, 37 °C; acid hydrolysis	80%	DHA/EPA in MG 76.2%	[157]
7	Sugar esterification	ARA		10 mg Lipase N-435 ^{**} , 30 mg IPXYL ^{****} , IPXYL:ARA, 1:1, mol/mol, 60 °C 12 h, followed by acid hydrolysis	83–85%	ARA-sugar ester	[158]
8	Inter-esterification	Cod liver oil concentration PUFA FFA fraction	74%	1 g corn oil, 0.5 g FFAs, 4 mL hexane, 7000 U lipase IM-60 ^{**} , 12 h; 40 °C		17.7% DHA/EPA in corn oil	[159]

^{*} Amano.

^{**} Novo.

^{***} Tanabe Seiyaku.

^{****} 1,2-*o*-Isopropylidene-D-xylofuranose.

Notwithstanding the low rates of PUFA esterification in the latter example, application of an appropriately selected lipase at a high activity rate can result in glycerol esterification of a concentrated PUFA FFA fraction from cod liver oil (Example 5). The PUFA FFA was prepared by urea complexation. By substitution of glycerol with 1,2 isopropylidene glycerol, which in effect blocks the 1 and 2 positions in the glycerol substrate, lipase can mediate selective esterification of PUFA FFAs to form PUFA monoglycerides (Example 6). A somewhat similar OH-blocking strategy, involving reaction 1,2-*o*-isopropylidene- α -xylofuranose with ARA is used in lipase-mediated esterifications to produce ARA sugar esters with potential applications in foods, cosmetics and pharmaceuticals (Example 7). Interesterification processes may be exploited to introduce, by substitution, new desired fatty acids into high volume oils. For example, *Rhizopus arrhizus* lipase has been used to produce cocoa butter substitutes from palm oil by substituting some of the stearate in the palm oil with oleate. Example 8 illustrates how this approach may be used to enrich corn oil with PUFAs originating from cod liver oil.

In conclusion applications of lipases to purify and/or otherwise transform PUFAs exploit well established and proven industrial lipase reaction systems, operated in aqueous or organic solvent media. However, for production of bulk PUFAs for dietary applications it appears that the microbial strains are already available to generate high concentrations of the desired PUFA in the right form (usually TG), such that appending a lipase conversion step is not necessary. In addition, the potential exists to select or manipulate producer strains or fermentation conditions to ensure minimize the need for substantial use of lipase procedures in PUFA enrichment. Hence, the main application of lipases with respect to PUFAs is likely to be for generation of non-natural esters of these products for use as pharmaceuticals or other synthetic bioactive compounds or their precursors.

15. Future outlook

The continuing accumulation and publication of evidence of the beneficial health effects of PUFAs has captured the attention not only of the medical community but also of a public at large, that is generally becoming aware of the importance of diet to general physical and mental wellbeing. While there was an understandable caution regarding the introduction of DHA and ARA into infant formula in the period since our first review [4] these supplements are now widely accepted by regulatory agencies and by the public. The major future growth market for PUFAs appears to be related to increasing PUFA content of the human diet, through dietary supplements, and perhaps through the introduction of PUFAs as transgenic plant and animal products. Clearly cellular and molecular methodologies are available to produce the desired PUFA components in

required amounts and in the appropriate conjugated forms (i.e. TGs, PLs) and conjugated at the desired glyceride position(s).

Natural or recombinant microbial systems are advantageous in that they can be tailored to produce PUFA oils of almost any defined composition and in fermentation processes, which facilitate ease of product recovery and purification. Microbial production processes are not weather-dependent or sensitive to diseases, and high oil production rates have already been demonstrated. The microbial process can also be manipulated to generate different conjugated forms. The technology is available and is already being exploited commercially for ARA and DHA production.

Areas for future research related to production of single cell oils include:

- further development of *Schizochytrium* strains to selectively produce higher levels of either ARA or EPA;
- further isolation of new thraustochytrids and screening for novel PUFA-related properties;
- further screenings and isolations of *Mortierella*-like species with novel PUFA-related properties;
- investigation of the properties and potential applications of genetically engineered heterotrophic algae, created from phototrophic species;
- generation of mutated strains producing novel PUFA intermediates and investigation of the potential bioactivity properties of the intermediates and their analogues;
- further development of lipases to better differentiate between PUFAs as substrates.

While recombinant system development is not as far advanced with respect to production of PUFAs in transgenic plants and animals, it is believed the technical methodologies exist. Hence, there is potential to alter the natural fatty acid profile to increase amounts of desired fatty acids and decrease undesired fatty acids. Indeed it is also considered possible to manipulate the fatty acid spectrum in selected tissues or parts of the plant which are amenable to harvesting and recovery. In the longer term it is likely that designer plant oils, containing PUFAs, will emerge from transgenic plants for addition to animal milks, infant formulae and other foods and feeds. With respect to animals there is potential to increase levels of expression of desaturase genes to greatly increase levels of desired PUFAs and in specific fluids, for example, in animal milk, which will facilitate direct use or incorporation of the milk into human foods. In addition, production of PUFAs in milk will facilitate PUFA recovery and purification for diverse applications.

Thus, advances in genetic and cellular methodologies, leading to better production systems, will ultimately make up for the shortages of these key dietary components available to the world's population and should contribute substantially to improving human health.

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