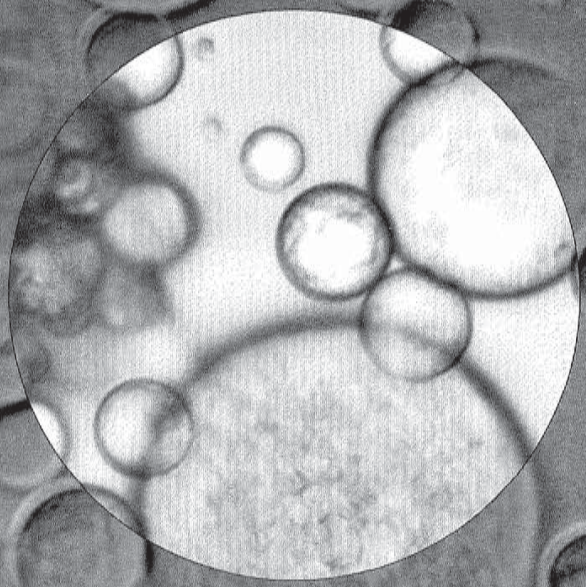
Single Cell Oils



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Single Cell Oils

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Dedication

To the memory of David Horrobin, 1939–2003, a scholar, a pioneer, and an inspiration to many.

Preface

Single cell oils (SCO) have come of age. They have become accepted biotechnological products fulfilling key roles in the supply of the major very long chain polyunsaturated fatty acids (PUFA), now known to be essential for infant nutrition and development. But their acknowledgment as being potential sources of oils and fats has been a slow process. Many critics in the early years of SCO doubted whether they could ever be produced at a reasonable price; even if they could, there were grave doubts as to whether SCO would be accepted by the general public. This was in spite of the "general public" having no apparent objection to consuming bacteria and yeasts as part of their everyday diet in the form of yogurts, cheeses, beers, and sourdough breads. When the product is good, the public will buy it; when the product is essential, the public will line up to buy it; and when our babies need the product, the line is likely to be a very long one indeed.

SCO are the edible oils extracted from micro-organisms—the single-celled entities that are at the bottom of the food chain. The best producers with the highest oil contents are various species of yeasts and fungi with several key algae also able to produce high levels of nutritionally important PUFA. Interest in SCO, as they have now become known, stretches back for over a century. Attempts have been made to harness the potential of various organisms, especially during the two world wars, in order to produce much needed oils and fats. Attempts have also been made to produce substitute materials for some of the major oilseed crops and even to produce a superior type of cocoa butter material. But it has been their potential to produce PUFA that has now galvanized the current interest in these SCO as oils rich highly desirable fatty acids essential for our well being and not readily available either from plants or animals.

This monograph has arisen from a symposium organized by David Kyle for the American Oil Chemists' Society in May 2003 that covered many of the ongoing projects in this area. It echoes two earlier conferences of the AOCS, the first in 1982 in Toronto and the second in Chicago in 1992, also organized by David Kyle. Over the intervening years, the position of SCO has become much more secure. Processes that were just "twinkles in the eye" in 1992 now exist as commercial realities; SCO production processes occur not only in the United States, but also in Europe, Japan, and China. Interest in them is widespread and the prospects of producing a complete range of PUFA is within our grasp. Whether the next decade or so will see SCO being overtaken by oils coming from genetically engineered plants, as has been predicted by some, will remain a tantalizing prospect. The future, as always, will be awaited with interest. In the meantime, SCO are here and available.

Zvi Cohen
Colin Ratledge

January 2005

Contents

Preface v

Chapter 1 **Single Cell Oils for the 21st Century**
Colin Ratledge 1

Chapter 2 **Arachidonic Acid-Producing *Mortierella alpina*: Creation of Mutants and Molecular Breeding**
Eiji Sakuradani, Seiki Takeno, Takahiro Abe, and Sakayu Shimizu 21

Chapter 3 **Development of a Docosahexaenoic Acid Production Technology Using *Schizochytrium*: A Historical Perspective**
William Barclay, Craig Weaver, and James Metz 36

Chapter 4 **Searching for PUFA-Rich Microalgae**
Zvi Cohen and Inna Khozin-Goldberg 53

Chapter 5 **Arachidonic Acid: Fermentative Production by *Mortierella* Fungi**
Hugo Streekstra 73

Chapter 6 **Production of Single Cell Oils by Dinoflagellates**
James Wynn, Paul Behrens, Anand Sundararajan, Joe Hansen, and Kirk Apt 86

Chapter 7 **Production of Docosahexaenoic Acid by the Marine Microalga, *Ulkenia* sp.**
Thomas Kiy, Matthias Rüsing, and Dirk Fabritius 99

Chapter 8 **Alternative Carbon Sources for Heterotrophic Production of Docosahexaenoic Acid by the Marine Alga *Crypthecodinium cohnii***
Lolke Sijtsma, Alistair J. Anderson, and Colin Ratledge 107

Chapter 9 **Carotenoid Production Using Microorganisms**
Michael A. Borowitzka 124

Chapter 10 **Prospects for Eicosapentaenoic Acid Production Using Microorganisms**
Zhiyou Wen and Feng Chen 138

Chapter 11 **Safety Evaluation of Single Cell Oils and the Regulatory Requirements for Use as Food Ingredients**
Sam Zeller 161

Chapter 12 **Nutritional Aspects of Single Cell Oils: Uses and Applications of Arachidonic Acid and Docosahexaenoic Acid Oils**
Andrew Sinclair, Nadia Attar-Bashi, Anura Jayasooriya, Robert Gibson, and Maria Makrides 182

Chapter 13 **Down-Stream Processing, Extraction, and Purification of Single Cell Oils**
Colin Ratledge, Hugo Streekstra, Zvi Cohen, and Jaouad Fichtali 202

Chapter 14 **Supercritical Fluid Extraction of Lipids and Other Materials from Algae**
Jaime Wisniak and Eli Korin 220

Chapter 15 **The Future Development of Single Cell Oils**
David J. Kyle 239

Index 249

Chapter 1

Single Cell Oils for the 21st Century

Colin Ratledge

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Introduction

Single cell oils (SCO) might be defined as the edible oils obtainable from microorganisms being similar in type and composition to those oils and fats from plants or animals. This chapter aims to provide an introductory overview to SCO and to show that the current interest in their production and use comes from a long history of interest in the exploitation of microorganisms as sources of oils and fats. Without these early endeavors, it is quite possible that none of the current commercial SCO products on the market would have been developed, since the basic understanding behind the exploitation of microbial oils would have delayed for several decades. The key events that led to the transition of microbial oils from being more or less academic curiosities 30 years ago to being important nutraceuticals for inclusion in infant formulas have been the overwhelming evidence of the dietary significance of very long chain, polyunsaturated fatty acids (PUFA) coupled with the realization that there is no adequate or safe source of them from plants or animals. What were originally unusual microorganisms have now turned out to be extraordinarily important since these are the only realistic sources of these oils. The diversity of microorganisms is so great that it can almost be assured that these current products will not be the last ones that will be launched in the 21st century as SCO.

The Early Years

There has been interest in microbial lipids for over 125 years (1) and in exploiting them as alternative sources of oils and fats for human consumption probably since the early years of the 20th century. Paul Lindner, working in Berlin, Germany, appears to have been the first person to develop a small-scale process to make a fat using a species of yeast then called *Endomyces vernalis* and currently known as *Trichosporon pullulans* (2,3). Work on the prospects of using microorganisms as a source of oils and fats continued to escalate during the first four decades of the last century with a number of groups in various countries studying not only the process of lipid biosynthesis but also the factors influencing its accumulation. These early endeavors into microbial oil production were reviewed in considerable depth by Woodbine (3) and

this review possibly remains the most thorough that is available covering worldwide developments of the subject from its very inception up to the mid-1950s.

The problem, though, was that the oils and fats produced by oleaginous species of yeasts and fungi (the groups of microbes that are the highest producers are called the "oleaginous" species) were not too different from the oils and fats obtainable from plant seeds. As these microorganisms had to be grown in culture medium that contained glucose or sucrose as a source of carbon, which was derived from agricultural crops, the cost of turning one agricultural commodity into another (i.e., turning sugar into oil) was never going to be economically feasible as the cost of sugar is never more than about a quarter of most of the commodity plant oils such as corn oil, soybean oil, and rapeseed (canola) oil. Moreover, it is not a question of turning one ton of sugar into one ton of oil. Microorganisms are not that efficient; it takes about five tons of sugar to make one ton of oil. Instantly, it can be appreciated that either one must find some zero-cost carbon source or identify oils that exceed the prices of the usual commodity oils by a considerable margin.

Nevertheless, in spite of these obvious economic limitations, considerable work on the production of microbial oils took place from the 1920s up to the late 1950s. This laid some very important foundations to understand lipid production in microorganisms. In brief, it was established that:

(i) The number of microorganisms capable of accumulating oil more than about 20% of their biomass weight was relatively small in comparison with the total number of species.

(ii) The oil-accumulating microorganisms were mainly species of yeast and fungi; few bacteria produced much extractable edible oil.

(iii) The oil produced by these microorganisms was, like plant oils, mainly composed of triacylglycerols having component fatty acids (FA) that were, in almost every case, similar to what had already been recognized in plant oils.

(iv) Some algae were recognized that produced fairly high amounts of lipid, but this lipid tended to be more complex than those from the yeasts and fungi; they still contained the same FA that occurred in plant oils. Some PUFA were observed to be similar to those found in fish oils.

(v) Oil accumulation in the oleaginous microorganisms could be increased by starving the cells of a supply of nitrogen—or a nutrient other than carbon. The cells responded to the deprivation of a key nutrient by entering into a lipid storage phase in which excess carbon, still present in the growth medium, was converted into storage lipid materials. If the cells were subsequently returned to a situation where there was now nitrogen available, the oil reserves could be mobilized and rechannelled into cellular materials. Lipid accumulation was a stress-induced response with the oil being an intracellular storage, reserve material.

A typical profile for the accumulation of lipid in an oleaginous microorganism is shown in Figure 1.1. This shows that lipid accumulation in a microbial cell only begins when nitrogen is exhausted from the medium. The medium therefore has to be formulated with a high C:N ratio to ensure that nitrogen is exhausted while other

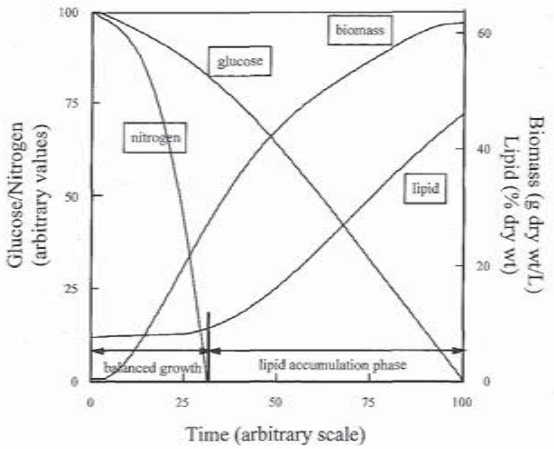


Fig. 1.1. Idealized representation of the process of lipid accumulation in an oleaginous microorganism. The composition of the culture medium is formulated so that the supply of nitrogen, which is usually an ammonium salt is growth limiting. After its exhaustion, cells do not multiply any further, but they continue to assimilate glucose (the usual carbon feedstock). This is then channelled into the synthesis of storage lipid (triacylglycerol) within the cells. The extent of lipid accumulation is dependent upon the individual microorganism; lipid contents may vary between 20 and 70% of the biomass.

nutrients, including carbon, remain in excess. In practice, this is about 40 to 50:1 (C:N) although the optimum ratio needs to be determined for each individual organism. To produce the greatest number of cells, the concentration of nitrogen and carbon may need to be increased while keeping them in the same proportion; this enables the balanced growth phase to continue until the maximum biomass density that the fermentor can sustain is reached before the lipid accumulation phase begins.

Although attempts were made in Germany during World War II to produce microbial fat to supplement the meager supplies that could be obtained from conventional sources (mainly animal fat with a little plant oil, there being little oilseed cultivation in Europe at this time and most plant oils were only obtainable from overseas), these efforts were limited. However, some oil-rich biomass production was achieved with fungi, mainly *Oidium lactis* (now *Geotrichum candidum*), being grown on waste lactose (from a cheese creamery) or agricultural waste material (4–7); this seems to have been fed, however, mainly to army horses by being formed into bricks using hay and straw (4). Some may have been included in soups and sausages for human consumption, but it was mainly viewed as a protein supplement rather than a source of fat. Although feeding the oil-rich fungus to army horses may sound rather trivial, the German army during this period had up to one million horses to support and using clearly unconventional sources of feed material was considered entirely reasonable.

Development of efficient large-scale production of microbial oils was limited by the availability of appropriate large-scale fermentors necessary to produce the biomass (microbial cells) to high densities (over 50 g dry wt/L). Laboratory-scale fermentors were relatively unheard of up to the 1950s, and industrial-scale stirred tank fermentors were rare. This lack of technology was demonstrated by the UK having to transfer the process for penicillin production in early 1940s (which had used static cultivation of *Penicillium chrysogenum* in adapted hospital bed-pans!), to the US which then had the only accessible stirred tank bioreactors in the world. This lack of technology was a clear limitation not only to microbial oil production but to almost all other microbial products that needed aerated, submerged cultivation systems. Obviously, some fermentors existed in many countries to produce beer and related materials, but these were for anaerobic production of microbial products and had no facilities for aeration or stirring. Moreover, most were open vessels and therefore were prone to airborne contamination.

The major stimulus to develop large-scale fermentation technology, and from it for the production of laboratory-scale fermentor units, was probably the advent of single cell protein (SCP) production that began in the late 1950s. Several petroleum companies, but principally BP Ltd of the UK, began to explore the conversion of *n*-alkanes, unwanted waste materials from the initial phase of fractionating petroleum oil, into edible biomass. Yeasts (especially *Yarrowia lipolytica*) were found that could grow rapidly on the alkanes, but, to achieve optimal conversion, stirred and aerated fermentors were essential. The ensuing biomass was rich in protein (about 50% w/w) and proved to be a useful major feed material for animals. As the manufacturers felt a little uneasy about describing their product as "microbial protein," the name SCP was coined as an appropriate euphemism to disguise the origins of the material.

This period ended because of unfavorable economics in 1975 with the price escalation of crude petroleum oil and the maintenance of the low price of soybean meal—the major competitor of the SCP. At the end of this period, the world had developed systems for submerged microbial cultivation to an unparalleled degree. Biotechnology had arrived, and not just for SCP production. Production of antibiotics, amino acids, and organic acids, such as citric acid, were now using sophisticated, stirred tank fermentation technology which had replaced cultivation of microorganisms in static cultures that primarily used shallow tray systems.

With the new technology becoming widely available (not forgetting the availability of laboratory-scale fermentors at a reasonable cost to allow research to be carried out at the 1–2 L level) interest in producing microbial oils once more re-emerged in the mid-1960s (8,9). However, enthusiasm for producing such products had largely waned since plant seed oils were now extremely cheap and there seemed little if any prospect of producing oils from other sources that could rival their price. There were, though, some prospects of producing some microbial oils (10–12) that were not readily available from conventional plant sources, but these ideas were still embryonic and lacked focus because the market for such materials was very uncertain. It is pertinent to point out that the examination of microorganisms carried out by Robert Shaw in the

early 1960s was focused on identifying possible sources of arachidonic acid (AA, 20:4n-6)—not for use in human nutrition (for which nothing was known at that point) but as a chicken-flavor material! Only after the work had been done was it realized that chicken flavor was not due to AA but to some entirely unrelated compound. The work of Shaw, however, proved invaluable for identifying microorganisms that might be used for the production of various long chain PUFA.

The other main development that occurred in the early 1960s, and was of considerable importance for the study of microbial oils, was the development of gas chromatography (13,14). Previously, FA analysis had been laborious and tedious and also required relatively large amounts of material. Gas chromatography altered all this; almost instantly one could analyze a number of oils and fats for their component FA and, moreover, use just milligram amounts of material. The stage was therefore set for a reexamination of microorganisms as potential sources of oils and fats; this can be seen from the seminal work of Bob Shaw mentioned previously and carried out from about 1960 to 1964.

Developments in the Last Quarter of the 20th Century

Although work in the author's laboratory (15–18) had been able to consolidate the mechanism of oil accumulation in yeasts being grown in laboratory fermentors using both batch and continuous fermentations and to confirm the approximate conversion efficiency of the starting substrate (glucose) to the product (triacylglycerol oil), there was, however, no clear target of which oil would be appropriate to consider for development. It was then brought to the author's attention that there might be a small niche market for an oil rich in γ-linolenic acid (GLA, 18:3n-6).

A Process for GLA Production

In the mid-1970s, GLA was available only as a minor component (about 9% of the total FA) of evening primrose oil (*Oenothera biennsis*), but nevertheless this oil was considered efficacious to relieve many symptoms and even for the treatment of multiple sclerosis—a claim that has long since been discounted—by virtue of its content of GLA. At the time, evening primrose oil commanded a price of about $50 per kg when most commodity plant seed oils were fetching less than a hundredth of this. Instantly, the prospects of a commercially viable SCO were recognized since it was known that there were microorganisms that synthesized GLA, and the work of Shaw (10–12) had established its consistent occurrence in a group of lower fungi known as the Zygomycetes.

Research carried out in the author's laboratory established that one member of this group was entirely suitable for producing an oil rich in GLA using large-scale submerged fermentation technology and commercialization of the process then followed with the first oil being produced in 1985 (19).

The first SCO was thus produced using *Mucor circinelloides* grown in large-scale fermentors of 220 m^3 (55000 US gallons). It was run by J. & E. Sturge Ltd at Selby,

North Yorkshire, UK, who normally used their skills in fermentation technology to produce citric acid using another fungus, *Aspergillus niger*. The oil was sold under the trade name of Oil of Javanicus and also as GLA-Forte that was used by one retailer of the oil. It achieved some limited penetration of the over-the-counter, food supplement market. By the time the process closed down in 1990, primarily due to a change in ownership of the company (to Rhone-Poulenc Ltd), falling prices in evening primrose oil, and the advent of borage oil as a cheaper alternative source of GLA, about 50 tons of material had been produced. Each run of the fermentor produced about 10 tons of biomass from which about 2 to 2.5 tons of oil could be extracted (see Chapter 13). A more detailed account of the GLA-SCO process is available (19).

The fungal oil, although superior to evening primrose oil in all respects—higher content of GLA (Table 1.1), higher stability to oxidation, absence of high levels of competing fatty acids such as linoleic acid, lower content of herbicide and pesticide residues—nevertheless had difficulty being sold to a public (mainly in the UK and some other European countries) that wanted evening primrose oil. Something that was superior to evening primrose oil but was not called "evening primrose oil" was viewed with suspicion even though marketing publicity carefully eschewed mentioning the microbial origins of Oil of Javanicus.

Although this first SCO failed to bring in a reasonable profit for the producers, nevertheless it was a significant milestone in the development of SCO. Its arrival encouraged other companies in other countries to explore the possibilities of using microorganisms as sources of similar and even more expensive oils and fats. Targeting of potential oils for niche markets was, however, still critical. A process related to the GLA-SCO process in the UK was developed in Japan by Idemitsu Kosan Co. Ltd, Tokyo, Japan using *Mortierella isabellina* and possibly also *Mort. ramanniana* (20). The oils produced were, however, much lower in GLA content than the oil produced by *Mucor circinelloides* (Table 1.1) although each fungus had about

TABLE 1.1
Fatty Acid Profiles of Fungi and Plants Used Commercially for γ-Linolenic Acid Production

	Relative % (w/w) of Major Fatty Acids									
	Oil Content (% w/w)	16:0	16:1	18:0	18:1	18:2 (n-6)	18:3 (n-6) GLA	18:3 (n-3)	20:1	22:1
Mucor circinelloides[a]	25	22	1	6	40	11	18	—	—	—
Mortierella isabellina[b]	~50	27	1	6	44	12	8	—	0.4	—
Mortierella ramanniana[b]	~40	24	—	5	51	10	10	—	—	—
Evening primrose	16	6	—	2	8	75	8–10	0.2	0.2	—
Borage	30	10	—	4	16	40	22	0.5	4.5	2.5
Blackcurrant	30	6	—	1	10	48	17	13	—	—

[a]Oil of Javanicus, citric acid produced by *Aspergillus niger*.
[b]Production organisms used by Idemitsu Co. Ltd, Japan. Oil contents of cells uncertain but approximate levels indicated.

twice the oil content of the *Mucor*. Sales of these oils to the Japanese domestic market began in 1988 although it is not known how much was sold or even if the process(es) are still extant; if the processes have now ceased, as seems likely, again it is uncertain when this occurred.

A Process for a Cocoa Butter Equivalent Fat

Some interest was developed in the early 1980s with the possible production of a cocoa butter equivalent (CBE) fat using yeasts. Yeasts, unlike many molds and fungi, tend to produce only limited amounts of PUFA and some strains can have relatively high contents of stearic acid (18:0). For a successful CBE, it is necessary to produce an oil or fat that has roughly equivalent amounts of stearate, oleate, and palmitate all accommodated on the same triacylglycerol molecule preferably as *sn*-1 stearoyl, *sn*-2 oleoyl, *sn*-3 palmitoyl glycerol (Table 1.2). The main problem to achieve this goal was to increase the rather low content of stearic acid in yeast fat up to at least 25%. This was initially attained using an inhibitor of the Δ9-desaturase that converts stearic acid into oleic acid (21). However, the inhibitor used, sterculic acid, was found to be more expensive to use than could be tolerated by required price of the final product. Instead, mutants of a yeast, *Candida curvata* (now *Cryptococcus curvatum*) were produced that had altered activities of this desaturase and thus produced the same type of product without having to use an expensive inhibitor (22) (Table 1.2). The mutants that were produced were not entirely stable, however, when used in large-scale fermentors; it was preferred to use the original, wild-type yeast, which had already a higher natural level of stearic acid than most other yeasts as a possible production organism (23,24). The key procedure used to increase the level of stearic acid was to use a very low aeration rate so that the desaturases were limited in their activities by oxygen availability, which is a co-substrate for their activity.

TABLE 1.2
Fatty Acid Profiles of Cocoa Butter Equivalent (CBE) Single Cell Oils (SCO): Microbial Oils Used as CBE Compared with Cocoa Butter

	Relative % (w/w) of major fatty acids					
	16:0	18:0	18:1	18:2	18:3 (n-3)	24:0
Cryptococcus curvatus Wt[a]	30	15	45	5	0.5	2
C. curvatus NZ[b]	18	24	48	3	1	2
C. curvatus R26-20[c]	15	47	25	8	2	—
C. curvatus R25-75[c]	33	25	33	7	1	—
C. curvatus F33.10[c]	24	31	30	6	—	4
Yeast isolate K7-2[d]	26	25	38	6	1	1
Cocoa butter	23–30	32–37	30–37	2–4	—	—

[a]Wt, wild-type yeast (original strain).
[b]NZ, strain used in New Zealand. See Davies (23).
[c]Mutant strains produced with partial deletions of Δ9-desaturase. See Smit *et al.* (22).
[d]Isolated in New Zealand. See Davies (23,24).

In spite of achieving a good quality CBE (Table 1.2) that could be incorporated into chocolate at the permitted level of 5% of the total fat, giving improved characteristics over the use of a conventional plant-derived CBE (R.J. Davies, personal communication), the yeast process was abandoned as not being sufficiently cost-effective. The cost of cocoa butter, which had been up to about $8000 per ton when the research work had begun, had fallen by the late 1980s to about $3000 per ton. Since a CBE could fetch only about 60% of this price, this left insufficient profit for the process to proceed beyond the pilot-scale level and an initial, one-off large-scale run at 250 m^3 (23). This was in spite of the process using virtually zero-cost lactose as feedstock with the lactose arising from the cheese creamery processes in New Zealand where there is so much of it that there are severe problems in ensuring its environmentally-friendly disposal!

Also taken into consideration when deciding to abandon this yeast CBE-SCO project was the uncertainty about the chocolate industry using the product even in confectionery products (e.g., for cakes, toppings, etc.) rather than in chocolate for direct consumption. Unease at using a "microbial fat" in chocolate products that depend very much on marketing images for high sales was a telling factor. Thus, with its target market being uncertain, the presence of adequate, alternative sources of other CBE, namely from palm oil fractionation, and the apparent low profitability of the microbial process, another SCO program was terminated.

SCO for the 21st Century

The Quest for a Docosahexaenoic Acid-Rich SCO

Having established that microorganisms could produce high quality oils and fats—though admittedly at a price—it was then a question of identifying which, if any, possible market might be exploited by these materials. Top consideration had to be given to oils that would be appropriate for human consumption rather than for animals, since these would be the markets able to command the highest prices as had already been seen with the GLA-SCO. At the same time, oils that could not be readily obtained from plant or animal sources would give an additional advantage to a microbial route of production, as the ensuing oil would then be free of serious competition. With these general considerations in mind, the work on the nutritional benefits and effects of the very long chain PUFA found in fish oil was of major importance.

There had been a steady investigation of the possible dietary benefits of fish oil since the pioneering work of Sinclair in the 1940s (25). However, the major findings that received international recognition arose from reports from Danish scientists investigating the reasons why cardiovascular problems seemed nonexistent, or at least significantly less, in Greenland Eskimos compared to other populations in spite of the very high intake of fat by the Eskimos (26). A low incidence of heart disease in other fish-eating populations of Norwegians and Japanese also helped to focus attention on the importance of docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3), being the two major PUFA of fish oils. By the 1980s, the importance

of both EPA and DHA for human nutrition was established and then, in the 1990s, the particular beneficial effects of taking DHA during pregnancy and for the nutrition of premature and newly-born, full-term babies began to appear (26,27). The presence of DHA and AA in mother's milk and their occurrence as the major FA of brain lipids and the retinal membrane lipids reinforced the concept that it would be highly beneficial if both these FA could be included in the diet of pregnant women and in infant formulas designed for the neonatal baby. A more complete account of the nutritional advantages of DHA and AA is covered expertly in Chapter 12.

Since it was DHA rather than EPA that was considered important, this meant that fish oils were not entirely satisfactory sources because all these oils contained both FA in roughly equal proportions (26); EPA was not, however, a "neutral" material that could be taken along with DHA. It appeared to interfere metabolically with the efficacy of DHA uptake and its incorporation into brain and retinal lipids and thus was contra-indicated (28). Tuna oil, though, appeared to be an exception to most other fish oils; it has a DHA to EPA ratio of 4:1 (26) which is about the same as occurs in mother's milk. But tuna oil was clearly in short, if not diminishing, supply and, in any case it did still have some EPA. The only solution that seemed appropriate was to embark on a very expensive process to fractionate DHA from fish oil. This would require several steps culminating in the use of preparative level high performance liquid chromatography (HPLC), which, by its very nature, was prohibitively expensive. No other source of DHA seemed apparent to nutritionists during the early 1990s.

Nutritionists, however, are not microbiologists and tend not to bother about microbial lipids or to know much about their composition except for recognizing that some marine microorganisms do contain DHA but usually in association with EPA. It did not seem apparent to any nutritionist in the late 1980s that microorganisms could be the key to providing a supply of DHA. It took someone who was aware of both the need for a good supply of DHA-rich oil and, simultaneously, had a knowledge of the FA composition of key microorganisms to be able to identify a potential microbial source of DHA. This was a major breakthrough and was pioneered by David Kyle and by the launch of his company, Martek Ltd, in the late 1980s that focused exclusively on developing a process using *Crypthecodinium cohnii* as the organism of choice for DHA production. *Crypthecodinium cohnii* was, though, already well known as a producer of an oil rich in DHA and of no other PUFA (29,30) but it was not apparent that it could be grown in very large scale fermentors to produce sufficient biomass to warrant considering it a commercial source of oil. Kyle and his colleagues, in a remarkably short period of time, demonstrated that this was feasible and went on to produce this oil which has since had a major impact on the infant nutrition market. A detailed account of the current process for producing DHASCO™ is given in Chapter 6.

An AA-Rich SCO

DHA, however, as indicated previously, was not the only FA that appeared to be important in infant nutrition. The other FA was AA (28). By a happy coincidence, a

microbial source of AA was already known through the work of Professor S. Shimizu in Japan (Chapter 2) using the Zygomycetes fungus, *Mortierella alpina* (31,32). However, the use of this oil for infant nutrition had not been considered and, thus, the opportunity of exploiting this technology independently of the Japanese work was then undertaken, again by Martek.

Martek was the only company that recognized what was needed by the infant formula market, by way of very long chain-length polyunsaturated FA (VLCPUFA), and knew how to obtain them. The foresight of the company was the recognition that both DHA and AA could have huge markets even if only perhaps 10% of all newly born babies were fed enriched infant formulas and even if only 0.1% of the weight of the formula might be DHA/AA. Multiply 0.01% of all infant formulas that are produced in the USA and Europe (not to mention in the 60+ other countries in which the product is now sold) and the potential of the SCO for this market can be quickly appreciated.

Microbial production of oils rich in both these FA are the main SCO in current production. Both processes were developed by Martek, although the one for AA was further developed by DSM (Chapter 5) working under license from Martek. Both processes began at a commercial level in the 1990s (33,34), and both are set to continue their expansion during the remaining years of this decade. In all probability, they will continue to dominate the market for both DHA and AA for some time, as it is highly unlikely that the demand for these VLCPUFA will diminish. Indeed, all the indications are that the demand for both FA will continue to grow until possibly there will be no infant formulas being produced in Western countries that will be without both materials. The only change in this would be the rather unlikely event of a significantly higher proportion of mothers choosing to breast-feed their babies rather than opting for formula-feeding.

The FA profiles of the commercial SCO are given in Table 1.3.

Other Sources of PUFA-SCO

DHA-Rich Oils. Not unexpectedly, once the DHA-SCO and AA-SCO oils were announced, other possible microbial sources of these materials were examined. An account is given in Chapter 3 of the process developed by OmegaTech Ltd, Boulder CO, to produce an oil rich in DHA using a species of *Schizochytrium* (35). Briefly, the oil produced was not a "DHA-only" oil but had about 20% of the DHA content as docosapentaenoic acid (DPA; 22:5n-6). This latter FA, although not of the same n-3 family of FA as DHA, is metabolically neutral and does not detract from the efficacy of uptake of DHA into key brain lipids; it does not add to the DHA content of the oil and, to this extent, diminishes the overall efficiency of DHA production in the organism. However, by the time this process was fully launched, the market for a DHA-only oil had been established by the *Crypthecodinium* oil and this has proved to be an unimpeachable position. Nevertheless, the *Schizochytrium* oil looks likely to be less expensive than the former oil, perhaps being half or even less the price as the organism grows about four times faster and also to very high cell densities: cell dry weight values of over 200 g/L, attained after 72 hours' growth, have been claimed (36).

TABLE 1.3
Fatty Acid Profiles (given as rel. % w/w) of SCO in Current Production[a]

A. Arachidonic Acid-SCO Processes Using *Mortierella alpina strains*

	14:0	16:0	18:0	18:1	18:2	18:3 (n-6)	20:3 (n-6)	20:4 (n-6)	22:0	24:0
DSM process[b]	0.4	8	11	14	7	4	4	49	—	1
Wuhan Alking process[c]	0.2	6.3	2.2	3.7	4.0	1.6	—	70.2	2.7	5.3

B. Docosahexaenoic Acid-SCO processes

	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3 (n-3)	20:3 (n-6)	22:5 (n-6)	22:6 (n-3)
Martek process[d] (DHASCO™)	4	20	18	2	0.4	15	0.6	—	—	—	39
OmegaTech process[e] (DHASCO-S)	—	13	29	12	1	1	2	3	1	12	25
Nutrinova process[f] (DHActive)	—	3	31	—	1	—	—	—	—	11	45

[a]For other abbreviations see Table 1.2.
[b]See Chapter 13.
[c]*Source:* Reference 43.
[d]Uses *Crypthecodinium cohnii*, see Chapters 6 and 13.
[e]Uses *Schizochytrium* sp., see Chapters 3 and 13.
[f]Uses *Ulkenia* sp., see Chapter 7.

The marketing (but not production) of the *Schizochytrium* oil, which was originally known as DHAGold but is now named DHASCO-S, is complicated by OmegaTech now being owned by Martek. Thus the senior company can chose to preserve the infant formula market for its own *Crypthecodinium* oil while exploiting other opportunities for the sale of the *Schizochytrium* oil. Such markets could well include feeding of farmed fish. Currently about 5 tons of fish meal are needed to bring one ton of fish to maturity in these fish farms. Clearly, this is nonsustainable and alternatives to fish meal are now actively being sought. Since the key ingredient of fish meal for growth and development, especially in the very earliest stages of fish growth, are the VLCPUFA, an alternative source of DHA would be extremely attractive. Although the costs of producing *Schizochytrium* biomass (for fish feeding one need not extract the oil but, instead, the whole biomass can be used) are considerably less than producing *Crypthecodinium* biomass, it would still appear to be more costly (possibly double) than fish meal itself. Nevertheless, it is a sustainable source of DHA. If it ultimately turns out not to be too prohibitive in price, governments or regulatory agencies may then chose to ban fish meal, or to at least place a moratorium on its use, in favor of a sustainable, alternative source.

A further reason for a move away from using fish meal for fish feeding is the presence in fish meals of various residues of man-made pesticides that have entered

the world's oceans and seas. These include dioxins and polychlorobiphenyls (PCB) as well as organo-heavy metals, including mercury compounds. Already the presence of such materials is too high to allow fish oils to be given as dietary supplements to infants in the U.S.

Further markets for SCO are also likely to be developed for other food uses and for which either the oil itself or the biomass could be used. We have already seen the incorporation of *Schizochytrium* biomass into poultry feed to bring about DHA-enriched eggs, which have been a minor marketing success (37). DHA-enriched milk and milk-derived products (cheeses, yogurts, etc.) and other food products are obvious extensions of this concept. It may be expected over the next decade or two that there will be a growing appreciation of the need for PUFA, such as DHA and perhaps AA, in adult nutrition in addition to their use in infant foods. The development of a whole range of DHA-supplemented foods, from margarines to salad dressings, would then be entirely feasible. The use of oils or biomass from organisms such as *Schizochytrium* is then bound to rise, and rise quite sharply, should these predictions be fulfilled.

It is also evident that further microbial sources of DHA are already being developed and considered as additional commercial sources of DHA-rich oils and DHA-rich biomasses to meet the expected increases in the market size for PUFA. Possible processes using marine organisms referred to variously as *Ulkenia* or *Labyrinthula* are under development in Japan (38,39) and in Germany. The latter process is reviewed in Chapter 7. The organisms being used are similar in a number of features to *Schizochytrium* spp. (40,41) and their oils, like that of DHASCO-S, always contain a significant proportion of DPA (42) (Table 1.3), which further emphasizes the similarity of this group of organisms. Commercial establishment of other, alternative processes for DHA production will clearly benefit the public since this will give both choice and a competitive price for the product.

AA-Rich Oils. Alternative microbial sources of AA are also being sought. Already it is known that there is a process for AA production in China, operated by Wuhan Alking Bioengineering Co. Ltd, using a new strain of *Mortierella alpina* (43). This process appears to operate at the 50–100 ton level (50,000–100,000 L). Work also appears to be ongoing to identify new organisms of interest for AA production: A new strain of *Mortierella alliacea* has been reported with contents of AA similar to those found in *M. alpina* of over 40% (44), and recent work (reviewed in Chapter 4) has found a new phototrophic algae, *Parietochloris incisa,* that has the highest content of AA of any phototrophically grown alga at over 40% of the total FA.

The overall activity in these areas to identify new, and possibly, improved sources of DHA and AA implies considerable economic potential in these processes. The lucrative nature of the markets will therefore continue to attract further interest from established biotechnology companies, and perhaps even pharmaceutical companies, all wishing for a share of the revenue.

PUFA-SCO for Clinical Applications. Clinical applications of the VLCPUFA seem, at the moment, to be restricted to the use of EPA rather than DHA or AA, which are now regarded as nutraceuticals or dietary supplements. Currently EPA is produced expensively by fish oil fractionation and chromatography, although possible microbial sources of it are under active consideration in a number of laboratories around the world (Chapter 10). The potential market size for EPA is difficult to estimate since applications of this PUFA seek to alleviate or cure various illnesses, including schizophrenia, bipolar disorder, certain cancers, Alzheimer's disease, and atherosclerosis, which are usually treated by expensive pharmaceutical drugs. It is worth pointing out that pharmaceutical companies have a vested interest in maintaining their position as the sole providers of the expensive medications for the treatment of these illnesses and disorders. Pharmaceutical companies will not encourage clinical and medical practitioners to prescribe, or suggest consumption of, a simple, over-the-counter FA that, although expensive, will nevertheless be much cheaper than a pharmaceutical drug.

There is no doubt that EPA is a very useful anti-inflammatory compound and can be given safely to many types of patients (Chapter 10). Nevertheless, problems about its source remain. Fish oils, for reasons discussed previously, are unlikely to be a satisfactory long-term source of EPA; for this reason alone alternative microbial sources would seem to be the preferred option. At the moment these sources appear to be photosynthetic algae (Chapter 10), although it would seem quite likely that microorganisms could be found that would be able to grow heterotrophically and thus parallel the situation with *C. cohnii* and other organisms being used for DHA production. Heterotrophic cultivation of microorganisms, although requiring a fixed carbon source and more expensive equipment, has a much higher productivity than phototrophic cultures that more than offsets these disadvantages. The problems in identifying an appropriate source of EPA should not be underestimated, since researchers have been trying to identify such a source for at least the past 5 years. It should, however, be noted that when *Mortierella alpina* is grown at a low temperature and supplemented with α-linolenic acid (18:3n-3) it can produce EPA instead of AA (Chapter 2). However, the process would appear to require a lengthy cultivation period (45), thereby increasing the costs of the oil substantially.

Other PUFA-SCO for clinical use, and perhaps for dealing with specific metabolic disorders, await market opportunities. Prospects of producing a variety of other PUFA, beside DHA, AA, and EPA, are discussed in Chapter 2 in which various mutants of *Mort. alpina* have been produced that synthesize useful amounts of stearidonic acid (18:4n-3), dihomo-γ-linolenic acid (20:3n-6), eicosatrienoic acid (also known as Mead acid; 20:3n-9), and eicosatetraenoic acid (20:4n-3). Of these, only stearidonic acid can be obtained from a plant source (*Echium*). In addition to these FA, DPA which occurs in the DHA-rich oils from *Schizochytrium* spp., is thought to be produced by Nagase-Suntory Co. Ltd in Japan. Whether this is produced as a by-product from the fractionation of the *Schizochytrium* oil or is produced using a specific organism, such as the novel labyrinthulid isolate that was recently reported to pro-

duce DPA as its sole VLCPUFA (46), is unknown. The isolate mentioned, though, needs to be cultivated on soybean oil to achieve DPA production; growth on linoleic acid or α-linolenic acid gives very low yields (46).

The applications of these unusual oils, either for treatment of various disorders or as dietary supplements, remain uncertain. For the first time, however, sufficient amounts of them can be produced by microbial technology that allows their evaluation to take place. It would not be too surprising if one or more of these VLCPUFA might be found to be beneficial for the treatment of certain conditions, in which case their large-scale production would quickly follow.

SCO and Competition from Genetically Modified Plant Oils

While future prospects for the continued production of various SCO look extremely strong, there is the undoubted prospect that one or more of the current SCO may be produced in plants at some future stage. Genetic manipulation of plants for improved characteristics has long been underway, and there are now several major industrial companies engaged in attempting to clone key genes into agronomically important plants to convert the existing FA of the oilseeds into AA, EPA, or DHA. Since none of these FA occurs in an agricultural crop, it is necessary for genes coding for various FA desaturases and elongases (Fig. 1.2, and Chapter 5) to be taken from a microorganism and inserted into the plant's DNA. These then have to be expressed (i.e., made to work); the resultant proteins have to be catalytically active (i.e., made to do the same job that they did in the original microorganism), but, in addition, they need to work only in the plant seed and work only at the time of oil accumulation in the seed. Thus the right genes have to be in the right place and work at the right time. If the new PUFA were produced throughout the entire plant—in the leaves, stem, and roots—the plant would probably be unable to grow properly.

There are, therefore, an enormous number of problems to be overcome for the successful genetic engineering of VLCPUFA into plants. Even finding the right genes in a microorganism is not an easy task. As pointed out by David Kyle in Chapter 15 of this book, the enzyme reactions to be carried out are complex: Both the desaturation and elongation reactions require more than one protein (Fig. 1.2). The simplest solution seems to have been for geneticists to try to clone an entire gene sequence from a microorganism; this sequence will then be able to code for the entire set of proteins needed for the synthesis of the new FA. Current progress in these areas has been frustratingly slow because of these difficulties. Further details concerning these problems are presented in Chapter 15 and in recent reviews written on this topic (47–49).

There is, however, an additional problem concerning the successful production of genetically modified (GM) plants that can synthesize significant amounts of VLCPUFA and that is the considerable metabolic cost to the plant for producing these materials. All FA are chemically reduced entities containing many methylene (-CH_2-) groups; for every acetate group that is used in synthesizing a FA chain, two molecules of the reductant, reduced nicotinamide adenine dinucleotide phosphate (NADPH), are

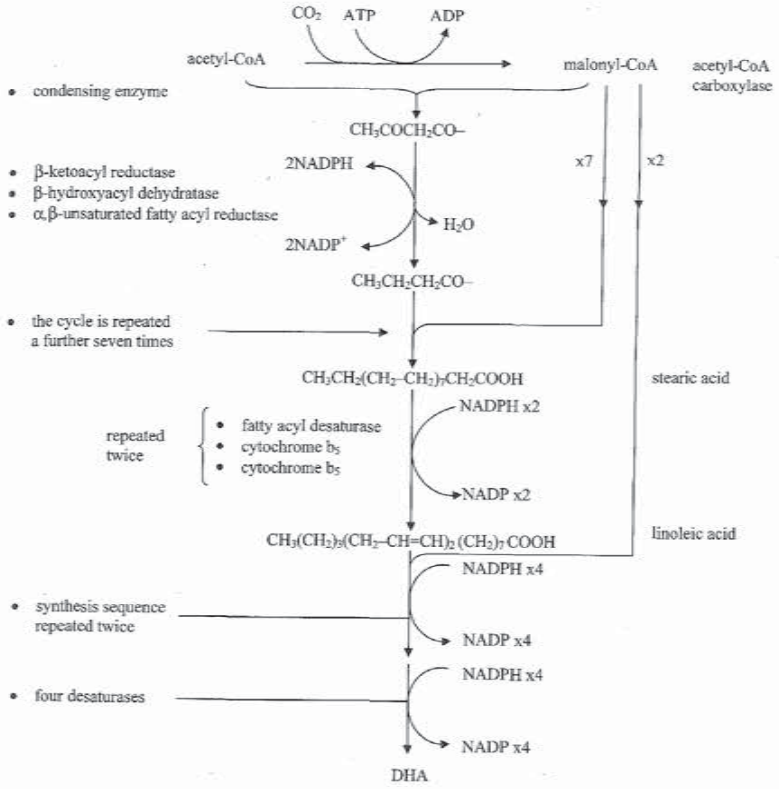


Fig. 1.2. Biosynthesis routes of two fatty acids: linoleic acid (18:2n-6) and docosahexaenoic acid (DHA, 22:6n-3) showing the requirements for reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the bio-reductant and adenosine triphosphate as the energy supply. The NADPH requirement for DHA biosynthesis is 80% greater than that needed by linoleic acid biosynthesis. (This assumes that DHA is being synthesized by a conventional eukaryotic fatty acid synthetase with accompanying desaturases.) In GM plants designed for DHA production, it is not clear how this additional supply of reductant will be provided or even if it *can* be provided without detriment to the well-being of the plant itself. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; and NADP, nicotinamide adenine dinucleotide phosphate.

needed to reduce the acetyl group (-CH_2CO-) into -CH_2CH_2- (Fig. 1.2). In addition, for every double bond that is introduced into the FA molecule *via* a FA desaturase, a further mol of NADPH is needed. Thus to make one mol of a FA, such as linoleic acid (18:2), 18 mol of NADPH are needed (2 × 8 for each condensation reaction + 1 for

each double bond) (Fig. 1.2). A further 8 mol of adenosine triphosphate (ATP) (as an energy source) are also needed for each condensation reaction, since ATP is needed for the conversion of acetyl-CoA into malonyl-CoA which is used at each stage of the FA biosynthesis sequence.

For the biosynthesis of DHA, the demand for NADPH rises to 26 mol/mol DHA, and 10 mol ATP are also needed. (This assumes that DHA is being synthesized *via* a conventional eukaryotic FA synthetase system and accompanying desaturases.) DHA synthesis thus requires an 80% increase in the supply of NADPH compared to the amount needed for linoleic acid synthesis. It is by no means clear where this extra NADPH is to come from, as the source of this reductant for FA synthesis in plants is unknown; in microorganisms the source of NADPH appears to be from malic enzyme reaction (Chapter 8, 50):

$$\text{Malate} + \text{NADPH} \rightarrow \text{Pyruvate} + CO_2 + \text{NADP}^+$$

Thus, even if all the genes for VLCPUFA synthesis can be introduced and temporally and spatially expressed in a plant, it may still be necessary to clone additional genes to produce the ancillary reducing power needed to achieve such synthesis. Ultimately, of course, the NADPH has to be generated from the photosynthetic reactions of the plant; most plants that grow in temperate climates are energy-limited by virtue of the availability of sunlight. A VLCPUFA GM-plant synthesizing appreciable amounts of, say, DHA will be even more energy-limited and, consequently, may not grow to the same extent as the original plant just producing linoleic acid. Energy will have to be taken away from other essential reactions of the cells and this may deplete the overall vigor of the plant. Alternatively, the plant may grow normally but then not divert sufficient energy into lipid synthesis, so that the production of DHA would be below expectations.

All this adds up to an enormous genetic engineering task. Whether a GM plant will be produced within the next 20 years that can synthesize useful amounts of DHA and the other PUFA, is of course, crystal ball-gazing. Several major industrial companies, including BASF, Monsanto, and DuPont, have extensive research activities in this area. It is therefore perhaps not a question of "if" PUFA-GM crops can be produced, but rather "when" they will be created.

When they are produced, the very relevant, ethical question can then be asked: Will the public accept such materials? Already there is a considerable ground swell of public opinion throughout Europe against all GM crops and this adverse opinion, which is not founded on the basis of any rational scientific argument, is unfortunately spreading into North America as well as elsewhere in the world. Possibly, by the time successful PUFA-GM crops have been created, governments of many countries may have banned their use for human food.

We thus stand at the brink of many exciting developments and some dilemmas. At least for the next two decades, it is more than likely that the supply of key PUFA (DHA, and AA, with EPA being a likely third prospect) will be met almost entirely

from microbial sources. Work to create GM crops has a long way to go before the first plants are produced that can synthesize useful amounts of VLCPUFA; it would then probably take a minimum of 5 years for these experimental plants to be grown commercially, assuming that all the difficulties in achieving high levels of gene expression have been solved. Of course, this always assumes that GM crops meet all the ethical requirements that are likely to be in place in 20 years.

Predicting future scientific successes (and failures) is a fool's game; after all, who could have predicted the current success of SCO at the beginning of the 1990s (51,52)? SCO have been successful because they are a product that is not obtainable from other sources and fulfills a primary demand for materials essential for the development and well-being of infants as well as adults. They are a product whose time has now come. How long they remain as the prime sources of these materials is completely uncertain, of course. Should GM crops be created that are viable, robust, and able to be cultivated successfully in the environment, and assuming that their cultivation will be permitted, then there is no doubt that these will be the cheapest source of the desirable PUFA oils. There is no way that a microbial process requiring a fixed source of carbon and a considerable input of energy to drive the production fermentors could compete with the low costs of production of an agricultural oilseed crop. However, there is still a very long way to go to achieve a GM crop that will produce the necessary amounts of VLCPUFA. As always, the future will be viewed with considerable interest.

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Chapter 2

Arachidonic Acid-Producing *Mortierella alpina*: Creation of Mutants and Molecular Breeding

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Introduction

The first trials of polyunsaturated fatty acid (PUFA) production with γ-linolenic acid (GLA, 18:3n-6) as the target were performed in the UK (1) and Japan (2), with *Mucor* fungi being used. Since then, various PUFA have been studied with the aim of effective production. For example, arachidonic acid (AA, 20:4n-6), dihomo-γ-linolenic acid (DGLA, 20:3n-6), and Mead acid (MA, 20:3n-9) are now commercially produced by using *Mortierella* fungi (3–6), and docosahexaenoic acid (22:6n-3), docosapentaenoic acid (22:5n-6), and eicosapentaenoic acid (EPA, 20:5n-3) by using marine microorganisms, Labyrinthulae and microalgae (6–10). Although the success in this area over the last 25 years has generated much interest in the development of microbial fermentation processes, manipulation of the microorganisms' lipid compositions requires new biotechnological strategies to obtain high yields of the desired PUFA.

In this chapter, we describe the recent progress in the breeding of commercially important arachidonic acid-producing *Mortierella alpina* strains, especially approaches for creating desaturase and elongase mutants with unique pathways for PUFA biosynthesis involving conventional chemical mutagenesis and modern molecular genetics. Such mutants are useful not only for the regulation and overproduction of valuable PUFA, but also as excellent models to elucidate fungal lipogenesis.

Arachidonic Acid-Producing *M. alpina* and Related Strains

The genus *Mortierella* has been shown to be one of the promising single cell oil (SCO) sources rich in various types of C20 PUFA (11,12), since several *Mortierella* strains were reported to be potential producers of AA in 1987 (13,14). In particular, several *M. alpina* strains have been extensively studied for the practical production of AA (15; Table 2.1). Some of them are now used for the commercial production of SCO rich in AA. Among them, *M. alpina* 1S-4 has a unique ability to synthesize the wide range of FA and has several advantages, not only as an industrial strain but also as a model for lipogenesis studies: (a) it is a highly oleaginous strain; (b) lipogenesis is simple and regulated; (c) it is one of the most well-studied microorganisms producing

Table 2.1
Arachidonic Acid Production by Various *Mortierella alpina* and Related Strains

Microorganism	Arachidonic Acid Yield/Cultivation Period	Scale	Year/Ref.
Submerged Culture			
Mortierella alpina 1S-4	3.6 g/L/7 d	5-L fermentor	1988 (16)
	3.0 g/L/10 d	2-kL fermentor	1989 (15)
	13 g/L/10 d	10-kL fermentor	1998 (17)
M. alpina 2O-17	2.5 g/L/5 d	5-L fermentor	1988 (18)
M. alpina CBS210.32	1.1 g/L/5 d	5-L fermentor	1989 (15)
M. alpina I-83	2.9 g/L/6 d	5-L fermentor	1989 (15)
M. alpina ATCC 32222	11 g/L/11 d	250-mL flask	1997 (18)
M. alpina ATCC 32221	11 g/L/16 d	500-L fermentor	1992 (19)
M. alpina UW-1	5.5 g/L/6 d	20-L fermentor	1995 (20)
M. alpina LPM 301	4.5 g/L/8 d	30-L fermentor	2000 (21)
M. alpina ATCC 42430	4.1 g/L/6 d	20-L fermentor	1996 (22)
M. alpina Wuji-H4	3.9 g/L/5 d	250-mL flask	1997 (23)
M. alpina DSA-12	3.3 g/L/6 d	500-L fermentor	1999 (24)
M. alpina CBS 343.66	1.0 g/L/8 d	5-L fermentor	1993 (25)
Mortierella elongata 1S-5	1.0 g/L/4 d	500-mL flask	1987 (13)
M. elongata SC-208	0.49 g/L/5 d	250-mL flask	1998 (26)
Mortierella alliacea YN-15	7.1 g/L/6 d	50-L fermentor	2001 (27)
Mortierella schmuckeri S12	2.3 g/L/3 d	14-L fermentor	1996 (28)
Mortierella sp. S-17	0.96 g/L/7 d	1-L flask	1990 (29)
Pythium irregulae ATCC 10951	3.1 g/L/8 d	250-mL flask	1999 (30)
Solid-State Culture			
M. alpina IFO 8568	13 g/kg-medium/20 d		1987 (31)
M. alpina CCF 185	36 g/kg-medium/21 d	300-mL flask	1993 (32)

PUFA; (d) it is able to incorporate and transform exogenous FA; (e) various desaturase and elongase mutants are available; (f) it is amenable to molecular genetic study; and (g) it can be used on an industrial scale.

The biosynthetic pathways for n-9, n-6, and n-3 PUFA in *M. alpina* 1S-4 are shown in Figure 2.1a. The main product of the strain, AA, is synthesized through the n-6 pathway, which involves Δ12 and Δ6 desaturases, elongase (EL2), and Δ5 desaturase. Depending on the conditions, the total amount of AA varies between 3 and 20 g/L (30–70% of the total cellular FA), with 70–90% of the AA produced being present as triacylglycerols (17,33,34). Cultivation of the strain under certain conditions also leads to a variety of PUFA being produced, for example: (a) lowering the growth temperature (<20°C) while simultaneously adding α-linolenic acid (ALA, 18:3n-3) to the medium results in the production of EPA (35,36); (b) the addition of Δ5 desaturase inhibitors, such as sesamin (37), to the growth medium causes an increase in DGLA (38); (c) utilization of C15 and C17 *n*-alkanes by the strain yields PUFA with odd numbers of carbons in their chains (total C17 and C19 FA reached over 95% of the

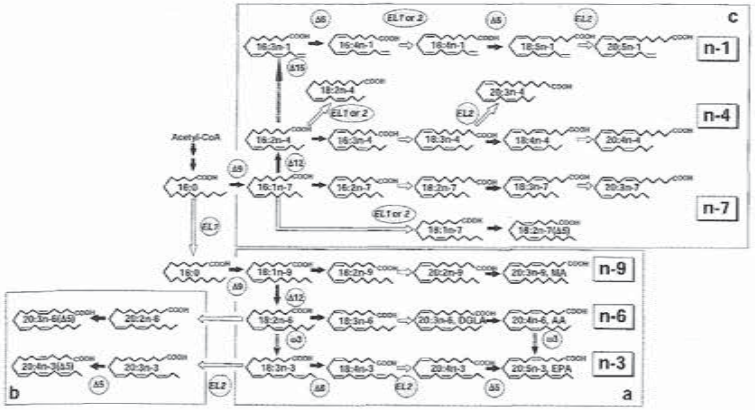


Fig. 2.1. Pathways for the biosynthesis of polyunsaturated fatty acids PUFA in *Mortierella alpina* 1S-4 and its mutants.

mycelial FA (39); and d) n-1 PUFA (20:5n-1, 20:4n-1, 18:4n-1, etc.) are produced when 1-hexadecene or 1-octadecene is the major carbon source (40).

Derivation of Mutants from *M. alpina* 1S-4

A wide variety of mutants defective in desaturases (Δ9, Δ12, Δ6, Δ5, and n-3) or elongase (EL1), or with enhanced desaturase activities (Δ6 and Δ5) have been derived from *M. alpina* 1S-4 by treating the parental spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (41). They are valuable not only as producers of useful PUFA (novel or already existing) but also for providing valuable information on PUFA biosynthesis in this fungus (5). The main features of these mutants grown on glucose and the biosynthesis of various types of PUFA by them are outlined in the following sections (see also Fig. 2.2).

Δ5 Desaturase-Defective Mutants

The FA profile of these mutants is characterized by a high DGLA level and a reduced concentration of AA (42). Production of DGLA by these mutants is advantageous because it does not require inhibitors, and the yield is relatively high (4.1 g/L, 42% in oil; AA content, <1%) (42–45). One of these mutants is used for commercial production of DGLA.

Δ12 Desaturase-Defective Mutants

The attributes of Δ12 desaturase-defective mutants include the absence of n-6 and n-3 PUFA and the high levels of n-9 PUFA, such as oleic acid (OA, 18:1n-9), octadeca-

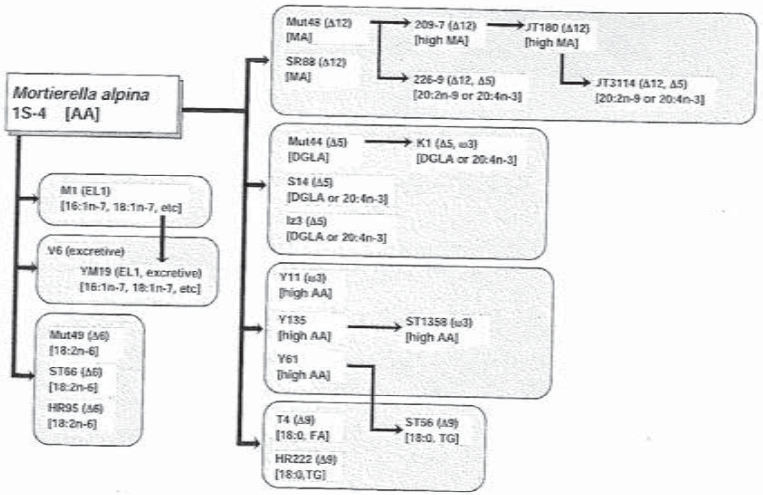


Fig. 2.2. List of the mutants derived from *Mortierella alpina* 1S-4. Symbols in parentheses indicate apparent mutation sites. Fatty acids in square brackets are major fatty acids produced by the mutants. Abbreviations: arachidonic acid, AA; dihomo-γ-linoleic acid, DGLA; free fatty acid, FA; Mead acid, MA; and triacylglycerol, TG.

dienoic acid (18:2n-9), eicosadienoic acid (20:2n-9), and MA, in their mycelia (46). Cultivation of these mutants under optimal conditions yields large quantities of a unique oil rich in MA. However, the addition of either n-6 or n-3 FA causes a rapid decrease in n-9 FA formation by these mutants and an increase in the AA or EPA level, respectively, because of the substrate specificity of Δ6 desaturase, which prefers linoleic acid (LA), ALA, and OA, in that order (47). Therefore, the same mutants can be used to produce an EPA-rich oil with a low AA level. ALA, when added exogenously (as linseed oil) to the medium, was efficiently converted to EPA, and produced a final mycelial EPA/AA ratio of 2.5 (47).

Double Mutants Defective in Both Δ12 and Δ5 Desaturases

These mutants accumulate 20:2n-9 in large quantities (48). However, the conversion of OA (endogenously produced from glucose) to 20:2n-9 hardly proceeds when the medium contains ALA for the same reason described previously. The ALA is preferentially converted to 20:4n-3 (49).

Δ6 Desaturase-Defective Mutants

Mutants synthesizing a high level of LA and low concentrations of GLA, DGLA, and AA are considered to be defective in Δ6 desaturase (50). These mutants are characterized by the accumulation of an eicosadienoic acid (20:2n-6) and a nonmethylene-interrupted n-6 eicosatrienoic acid (20:3Δ5). The latter PUFA is thought to be synthesized through elongation of LA and Δ5 desaturation, as shown in Figure 2.1B. In a similar manner, a nonmethylene-interrupted n-3 eicosatrienoic acid (20:4Δ5) can be produced from ALA added to the medium (50).

n-3 Desaturase-Defective Mutants

These mutants are unable to synthesize n-3 PUFA when grown at a low temperature (<20°C) (51,52). The wild strain usually gives the highest AA yield at 20°C, although a part of the AA formed is further converted to EPA and the resultant oil includes a small amount of EPA (*ca.* 3% in oil). Therefore, these mutants are superior to the wild strain when SCO with a relatively higher content of AA is needed (52).

Δ9 Desaturase-Defective Mutants

Stearic acid (18:0) is the main FA in the mycelial oil (up to 40%) produced by these mutants (53). However, Δ9 desaturase is not completely blocked. A total blockage would be lethal since low activity of the enzyme is necessary to introduce the first double bond at the ninth carbon (from the carboxyl end) of the FA chain to maintain cell viability (see next section).

Elongase (EL1 for the Conversion of Palmitic Acid to Stearic Acid)-Defective Mutants

The FA profile of the mutants with lowered EL1 activity is characterized by high levels of palmitic acid (16:0) and palmitoleic acid (16:1n-7), with small amounts of various kinds of n-7 and n-4 PUFA, which are not detected in the wild strain. The total content of these PUFA in the oil reaches about 30%. These PUFA are thought to be derived from the palmitoleic acid accumulated through the n-7 and n-4 pathways, respectively, as shown in Figure 2.1C (34,54). In a similar manner, n-1 PUFA can be produced from n-1 hexadecaenoic acid (16:1n-1) added to the medium (Fig. 2.1C) (34,55). Therefore, palmitoleic acid corresponds to OA, and the n-7, n-4, and n-1 pathways to the n-9, n-6, and n-3 pathways, respectively, in the wild strain. In Figure 2.1C, whether EL1 or EL2 elongates unsaturated C16 FA to the corresponding C18 FA is not made clear, because EL1 activity is not completely blocked.

Mutants with Enhanced Desaturase Activities

A mutant (209-7) with enhanced Δ6 desaturase activity was isolated from a Δ12 desaturase-defective mutant (Mut 48) by selecting colonies with high MA contents after mutagenesis (56). Δ6 Desaturase activity was increased 1.4-fold in this mutant, from which a mutant (JT180) with elevated Δ5 desaturase activity (3.3-fold) was obtained (Fig. 2.2). Cultivation of JT180 yields a large quantity of MA (2.6 g MA/L, 49% in oil) (57). This mutant is used for commercial production of MA.

Analysis of Enzyme Genes Involved in PUFA Biosynthesis in *M. alpina*

Δ9 Desaturase

The cDNA and genomic gene for Δ9 desaturase (Δ9-1) from *M. alpina* 1S-4 have been cloned (58). There is a cytochrome b_5-like domain linked to the carboxyl terminus of this desaturase, as can also seen for yeast Δ9 desaturase. The Δ9 desaturase of *M. alpina* 1S-4 exhibits 45% and 34% amino acid sequence similarity with those of *Saccharomyces cerevisiae* and rat, suggesting that the *Mortierella* Δ9 desaturase (Δ9-1) is a membrane-bound protein of the acyl-CoA type. The *Mortierella* Δ9-1 genomic gene has only one intron, with a GC-end occurring at the 5′-terminus instead of a GT-end, which is generally found in the introns of eukaryotic genes. The full-length cDNA clone was expressed under the control of the *amyB* promoter in *Aspergillus oryzae*, resulting in drastic changes in the FA composition in the transformant cells; the contents of palmitoleic acid and OA increased significantly, with accompanying decreases in those of palmitic acid and stearic acid.

Multiple Δ9-desaturases (Δ9-2 and Δ9-3) other than Δ9-1 are present in *M. alpina* ATCC 32222 (59) and *M. alpina* 1S-4 (60). Both Δ9-1 and Δ9-2 desaturate stearic acid to OA, whereas Δ9-3 desaturates a very long saturated FA (26:0) to the corresponding monosaturated FA (26:1n-9) (59). The genes encoding Δ9-2 and Δ9-3 are not transcribed as much as that encoding Δ9-1 in the wild strain. In the mutants defective in Δ9-1, the Δ9-2 gene is transcribed effectively at the same level as the Δ9-1 gene, whereas Δ9-3 mRNA is hardly synthesized in either the wild strain or the mutants (60). These results suggest that Δ9-2 acts as Δ9-1 in these mutants.

Δ12 Desaturase

A cDNA for Δ12 desaturase has been cloned from *M. alpina* 1S-4 (61), based on the sequence information on ω3 desaturase genes (from *Brassica napus* and *Caenorhabditis elegans*), that are involved in the desaturation of LA to ALA. The amino acid sequence of the Δ12 desaturase shows 43.7% identity, the highest match, with a microsomal Δ6 desaturase (from *Glycine max*, soybean), whereas it exhibits 38.9% identity with a microsomal ω3 desaturase (from soybean). The cloned cDNA was confirmed to encode a Δ12 desaturase, involved in the desaturation of OA to LA, from its expression in both *S. cerevisiae* and *A. oryzae*. The cDNA expression in *A. oryzae* allowed a fungal transformant to accumulate LA corresponding to over 70% of the total FA. Genomic Southern blot analysis of the transformant confirmed integration of this gene into the genome of *A. oryzae*. The *M. alpina* 1S-4 Δ12 desaturase is the first example of a cloned non-plant Δ12 desaturase.

Δ6 Desaturase

Based on the sequence information of Δ6 desaturase genes from borage and *C. elegans*, a cDNA encoding Δ6 desaturase (Δ6-1) involved in the desaturation of LA to GLA has been cloned from *M. alpina* 1S-4 (62). The predicted amino acid sequence shows similarity to those of the Δ6 desaturases of the other species, and contains a cytochrome b_5-like domain at the *N*-terminus; this is different from the *M. alpina* Δ9-1 and yeast Δ9 desaturase, which have the corresponding domain at the *C*-terminus. The full-length cDNA clone was expressed under the control of the *amyB* promoter in *A. oryzae*, and resulted in the accumulation of GLA to the level of 25.2% of the total FA. Coexpression of both Δ12 and Δ6 desaturase cDNA from *M. alpina* ATCC32221 in *S. cerevisiae* was also shown to cause the accumulation of GLA (63).

A second Δ6 desaturase (Δ6-2) is present in *M. alpina* 1S-4 (64). The amino acid sequence homology between Δ6-1 and Δ6-2 is very high (92%). The genes are both transcribed at different levels in *M. alpina* 1S-4. Usually, the Δ6-1 gene is transcribed much more highly (2- to 17-fold) than the gene for Δ6-2. Only the Δ6-1 is defective in the Δ6 desaturase-defective mutants obtained thus far by chemical mutagenesis (65).

Δ5 Desaturase

A cDNA encoding Δ5 desaturase has been isolated from two *M. alpina* strains (CBS 210.32 and ATCC 32221) and its function was confirmed by its expression in *S. cerevisiae* and canola (66,67). Expression in transgenic canola seeds resulted in the production of unique PUFA, taxoleic acid (Δ5,9-18:2) and pinolenic acid (Δ5,9,12-18:3), the Δ5 desaturation products of OA and LA, respectively. The deduced amino acid sequence of the Δ5 desaturase from the CBS strain exhibits a 22% identity with the Δ6 desaturase from the cyanobacterium *Synechocystis* and a 20% identity with the borage Δ6 desaturase. It contains a cytochrome b_5-like domain at the *N*-terminus like the previously mentioned Δ6 desaturases. These *Mortierella* enzymes are the first examples of cloned Δ5 desaturases. The *M. alpina* 1S-4 Δ5 desaturase genomic gene has 7 introns. Genetic analysis revealed mutated sites in the Δ5 desaturase genes from *M. alpina* 1S-4 mutants (Mut44, S14, Iz3, and M226-9; Fig. 2.2). The G nucleotides at the 566th and 903rd positions from the start codon in the Δ5 desaturase genes of Iz3 and M226-9 were replaced with A nucleotides, respectively. As a result, these mutations caused amino acid replacement of G189E and W301Stop in the Δ5-desaturases of Iz3 and M226-9, respectively (68). The C nucleotide at 10 bases upstream from the AG-terminal of the first intron was replaced with an A nucleotide in the Δ5 desaturase gene of S14. The Δ5-desaturase cDNA of S14 was 8 bp longer than that of the wild strain when the cDNA is compared. It was assumed that the new A nucleotide created by the mutation was recognized as a component of the new AG-terminal of the first intron and thereby caused a frameshift mutation (68). The Δ5 desaturase gene and its promoter region of Mut44 had no nucleotide replacement.

Elongase

A cDNA clone with a 957-nucleotide open reading frame encoding 318 amino acid residues has been isolated from *M. alpina* ATCC 32221 and expressed in *S. cerevisiae*

(69). The cDNA exhibits a 25% identity with the ELO2 protein (EL2) responsible for the elongation of saturated and monosaturated FA. Co-expression of this cDNA with that of *M. alpina* Δ5 desaturase in the yeast resulted in the conversion of exogeneously added GLA to AA *via* DGLA, as well as the conversion of n-3 octadecatrienoic acid (18:3n-3) to EPA; this suggests that EL2 plays a critical role in the elongation of both n-6 and n-3 C18 PUFA to the corresponding C20 PUFA, respectively. This is the first example of a cloned elongase.

NADH-Cytochrome b_5 *Reductase*

A cDNA clone with an open reading frame encoding 298 amino acid residues showing a marked sequence similarity to NADH-cytochrome b_5 reductases (CbR) from other sources (*S. cerevisiae*, bovine, human, and rat) was isolated from *M. alpina* 1S-4 (70). The corresponding genomic gene has 4 introns of different sizes in base pairs. According to the general GT-AG rule, these introns have GT at the 5′-end and AG at the 3′-end. The expression of the full-length cDNA in *A. oryzae* resulted in a 4.7-fold increase in ferricyanide-reduction activity involving the use of NADH as an electron donor in its microsomes. This *Mortierella* CbR was purified with a 645-fold increase in the NADH-ferricyanide reductase specific activity. The purified CbR preferred NADH to NADPH as an electron donor. The second CbR gene of the same fungus has also been characterized (71). Its cDNA and predicted amino acid sequence exhibit about 70% similarity to those of the first gene.

Cytochrome b_5

Cytochrome b_5 has been purified from *Mortierella hygrophila* IFO 5941 and characterized in some detail (72). Both the cytochrome b_5 genomic gene and cDNA from *M. alpina* 1S-4 have been cloned (73). The genomic cytochrome b_5 gene consists of 4 introns and 3 exons. A novel type of RNA editing, in which the cDNA has either a guanine insertion or adenine-guanine substitution at one base upstream of poly(A), is involved in the transcription process. The amino acid sequence of *M. alpina* 1S-4 cytochrome b_5 exhibits a 48, 40, and 39% identity with those of rat, chicken, and yeast, respectively, over 100 amino acids of the N-terminus. By contrast, there is no significant identity among these sequences over 30 amino acids of the C-terminus. The soluble form of the cytochrome b_5 reached 16% of the total soluble protein in *Escherichia coli*. The holo-cytochrome b_5 accounted for 8% of the total cytochrome b_5 in the transformants.

Genetic Manipulation of *M. alpina* Strains

Recently, transformation systems for *M. alpina* strains were reported by two laboratories (74–76). The first one utilizes the gene for hygromycin resistance. Using a homologous histone H4 promoter, *M. alpina* CBS 224.37 was successfully transformed to hygromycin resistance. The genetically stable transformants require inclusion of a homologous ribosomal DNA region in the vector to promote chromosomal integration (73).

The second one involves a ura$^-$ mutant lacking orotate phosphoribosyl transferase activity from *M. alpina* 1S-4. Transformation with a vector containing the homologous *ura5* gene as a marker, produced by microprojectile bombardment, resulted in stable transformants overexpressing the *ura5* gene and unstable ones showing orotate phosphoribosyl transferase activity comparable to the wild strain. The stable transformants retained the *ura5* gene originating from the transformation vector regardless of the culture conditions, while the unstable transformants easily lost the marker gene under uracil-containing conditions (76). Using this transformation system, overexpression of the gene encoding EL2 has been successfully performed (77). EL2 has been suggested to be the limiting step for AA biosynthesis (78). The resultant transformants yield more AA than the wild strain. However, these two transformation systems for the industrially important oleagenous *Mortierella* fungi need further improvement because the transformation frequency is still relatively low compared with those reported for other filamentous fungi, such as *Aspergillus*, *Neurospora*, and *Trichoderma*.

Conclusion

The unique transforming enzyme systems of *M. alpina* 1S-4 and its mutants can be used to obtain various PUFA of the useful n-9, n-6, n-3, n-7, n-4, and n-1 series from glucose or precursor FA added to the medium. They also make it possible to control the FA profiles of fungal mutants and to regulate the flow of glucose or exogenous FA to obtain a desired PUFA. Because of the simplicity of their metabolic systems, these mutants are potentially ideal models to elucidate fungal lipogenesis. Presently, studies on *M. alpina* and its mutants are focused on the molecular engineering of these enzyme systems. Thus, these *Mortierella* strains may be used not only for the molecular breeding of oleaginous microorganisms but also for the creation of transgenic oil plants with desired FA compositions.

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Chapter 3

Development of a Docosahexaenoic Acid Production Technology Using *Schizochytrium:* A Historical Perspective

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Introduction

Importance of Long-Chain n-3 Fatty Acids in Human Health

Recognition of the nutritional importance of the long-chain polyunsaturated n-3 fatty acids (LCPUFA), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), began emerging in the mid-1980s. Research during the previous two decades had indicated that, prior to the introduction of modern agricultural practices, human intake of these fatty acids (FA) was much higher; that populations whose intake of these FA was higher exhibited lower occurrence of chronic diseases including cardiovascular diseases, arthritis, asthma, and diabetes; that unique compounds called eicosanoids were made from these FA; and these eicosanoids had protective effects against the initiation and progressive development of these diseases (1,2).

In the 1980s, the major source of these LCPUFA in the human diet was from fish or fish oil capsules. Consumption of fish in North America was historically low, and fish oil capsules met with poor acceptance because of their inferior organoleptic (taste and odor) characteristics. Attempts to utilize fish oils as an ingredient in foods were also largely unsuccessful because of their strong fishy taste and odor problems. Attempts to improve the organoleptic characteristics of fish oil remained unsuccessful, and there were emerging concerns with the levels of environmental contaminants (PCBs, dioxins, and mercury) reported in fish (3) that still continue to this day (4).

It was recognized at the time that the primary source of n-3 LCPUFA in fish was not from biosynthesis by the fish, but rather from their diet, a result of the plankton they consumed. We hypothesized that if one could develop a way to economically cultivate a microbial strain particularly rich in n-3 LCPUFA, it would provide an acceptable source of these compounds that avoided the organoleptic and environmental contaminant problems found in fish and fish oils. Although fish oils contained both EPA and DHA, and much of the research demonstrating the importance of n-3 LCPUFA was conducted with fish oil, a decision was made to focus on the production of DHA initially. Emerging research indicated that while EPA had significant anti-inflammatory effects (5), DHA was important in infant nutrition (6), had positive

effects on blood lipids and cardiovascular health in adults (7), was more effectively retained in the human body (10 wk for DHA vs. only about 2 wk for EPA), and DHA could be readily retroconverted to EPA (8,9). These and other factors suggested that DHA might be a better form of n-3 LCPUFA to supply as a food ingredient or nutritional supplement for consumers.

Sources of n-3 LCPUFA

In the 1980s, marine microalgae were recognized as the primary microbial producers of n-3 LCPUFA. Bacteria and yeast were not known to produce n-3 LCPUFA, although later, a few strains of bacteria were discovered in the intestines of deep sea fish that could make EPA, but only the phospholipid form (10). Since phospholipids comprise a maximum of approximately 10–15% of the dry weight of microbes, a phospholipid producer of long-chain n-3 FA would not prove to be an especially economical producer of DHA for food uses. However, a few microalgae and fungi can accumulate up to about 80% of their dry weight as triaclyglycerols. Cost-effective production of DHA from microbes would require the use of microbial strains that could produce large amounts of triacylglycerols (11).

Although outdoor ponds and photobioreactors were the most commonly utilized production systems for microalgae at the time (12,13), the use of these systems was very expensive because of low productivities. Low cell concentrations require large quantities of water to be centrifuged to recover the biomass. There was also a lack of controls to maximize formation of the target product, and an inability to ensure food-grade production conditions in outdoor production systems. Although heterotrophic production of algae in large-scale fermentors had been proposed, it focused on primarily employing freshwater strains of microalgae that are not good n-3 LCPUFA producers (14). Additionally, since n-3 LCPUFA were associated with the photosynthetic membranes of microalgae, initially there was a bias against using a non-photosynthetic production system to produce these FA.

Two competitive processes to produce long-chain n-3 FA were under development at the time. Japanese researchers were attempting to modify a fermentation process for arachidonic acid (ARA, 20:4n-6) using the fungus *Mortierella* to enable it to also produce EPA (15). Process modifications included growing the fungus at low temperatures or chilling the harvested biomass for several days to induce the production of EPA. However, this resulted in very low overall productivity. The second competitive process involved culturing the slow growing apochlorotic dinoflagellate, *Crypthecodinium, via* fermentation (16). These authors believed that significant improvements in these two technologies would be necessary before they could enable the inexpensive production of DHA required for a cost-effective ingredient in foods.

Need for Alternative Technology Development

Recognizing the need for an alternative source of n-3 LCPUFA and reflecting on the state of the competitive technologies being developed at the time, the authors decided

to pursue the development of an alternative technology using a different set of assumptions. First, it was thought that there were no known strains of microalgae that could be developed to produce n-3 LCPUFA fatty acids effectively at a cost that could compete with fish oil. Development of a microbial production technology would require isolation and identification of new, natural overproducers of n-3 FA. Second, these strains would need to produce long-chain n-3 FA at relatively high temperatures, a property that was counterintuitive to the belief at the time that microalgae normally made these FA under cold conditions in an attempt to keep their membranes flexible and functioning. Third, the authors recognized the fundamental need to address the significant corrosion problems associated with growing marine microorganisms in conventional stainless steel fermentors. Others had grown marine microalgae in glass-lined fermentors to avoid corrosion. However, on a large scale these fermentors were very expensive, and only a few were available commercially around the world. Glass-lined fermentors only partially solved the problem, and ignored the associated issues with corrosion in downstream processing equipment (which cannot be glass-lined) and problems with disposal of waste high salt fermentation media at locations far from the ocean. Since the most likely production candidates (if they existed) would be marine strains, new strategies to cultivate them under very low salinity conditions would have to be developed to minimize the corrosive effects of seawater-like fermentation media.

After identifying all of the problems with alternative technologies, it was decided that the best way to rapidly, competitively, and comprehensively address all of these issues would be to utilize a bio-rational approach to strain collection/isolation/screening. Hopefully, this would best ensure enrichment and identification of strains with all or most of the desirable production characteristics and greatly facilitate technology development. This chapter describes the design and implementation of a bio-rational approach to microalgal technology development that resulted in the intense study and ultimate commercialization of products based on a unique group of microorganisms. Additionally, this chapter outlines the numerous benefits that result from the use of this type of approach to microbial technology development. Since development of this technology started in 1987, numerous researchers have recognized the biotechnological potential of this group of microorganisms (thraustochytrids) (17) and have contributed to the understanding of both their production potential (18–23) and their potential to produce compounds other than FA (24,25).

Bio-Rational Approach to Technology Development

A bio-rational approach first involves the identification of the most desirable characteristics that an ideal production strain should possess in terms of both the target production system and the target product. A strategy of isolating such a strain (if it exists in nature) is then developed drawing upon concepts derived from sciences such as ecology, physiology, biochemistry, and evolution. The ultimate goal of the proposed technology was to produce large quantities of DHA inexpensively from heterotrophic

microalgae in conventional stainless steel fermentors utilizing glucose as the carbon source. With this in mind, a bio-rational collection/isolation/screening program was designed to isolate microorganisms with the combination of characteristics outlined in Table 3.1. These characteristics were deemed desirable in a microorganism to be utilized for the economical production of n-3 FA. In a complementary fashion, the collection strategy was devised to collect microorganisms from habitats that experience a wide range of temperatures and salinities. Examples of these locations included micro-habitats in the following locations: marine tide pools, estuaries, and inland saline lakes, playas, and springs. The working hypothesis was that, in addition to their ability to grow at low salinities and high temperatures, some of these strains might be natural overproducers of DHA as an adaptation for their survival in these harsh environments, with DHA possibly playing a key role in helping to stabilize membrane function in rapidly fluctuating environments.

Implementation of the collection/isolation/screening program has been outlined in detail by Barclay (26). Briefly, water samples from target aquatic niches were run through a sandwich filtration system eliminating cells greater than 25 μm; polycarbonate filters containing biomaterial from 1–25 μm were placed on low and medium salinity nutrient agar plates and incubated at 30°C; clear and white colonies that were not yeast colonies were picked, cultured, and then analyzed for their FA profile and content by gas chromatography.

The initial results indicated that the bio-rational collection/isolation strategy had selected for two groups of microalgae or algae-like microbes, diatoms and thraustochytrids. The newly isolated diatom strains, however, primarily produced EPA and were relatively slow growing. Additionally, diatoms were known to have silica cell

TABLE 3.1
Targeted Production- and Product-Related Characteristics Selected for by the Collection/Isolation/Screening Program and the Bio-Rationale Behind Selection of These Characteristics

Characteristics Important for Production in Fermentors	Bio-Rationale
Capable of heterotrophic growth	Produce on inexpensive carbon source (corn syrup)
Unicellular (non-filamentous) and ≤25 μm in diameter	Minimize mixing energy needed in fermentor
Thermotolerant (grow above 30°C);	Higher temperatures equate to faster production
Euryhaline (grow especially at low salinity)	Minimize corrosion in the stainless steel fermentors
Characteristics Important for Utilization of Whole Cell Algae or Extracted DHA Oil in Foods	**Bio-Rationale**
High content of omega-3 highly unsaturated fatty acids	High content of target product
Preferably a low content of saturated and n-6 fatty acids	Undesirable in some applications
Preferably non-pigmented, white or colorless cells	Target is to be an invisible ingredient in foods

walls that might make them difficult to grow at high concentrations in fermentors because of problems with fragility and supplying silica to the fermentation medium.

Therefore, the authors decided to focus on the thraustochytrid strains. Thraustochytrids were relatively little known at the time from a biotechnological perspective. Thraustochytrids are microalgae or microalgae-like microorganisms. The earliest research on thraustochytrids placed them in the fungi because of their heterotrophic nature and superficial resemblance to chytrids (27). However, later analyses using molecular biology techniques demonstrated that thraustochytrids were not fungi, but instead were related to the heterokont algae (28). Prior to the late 1980s, most studies on thraustochytrids had focused on their distribution, taxonomy, ultrastructure, and physiology. Because thraustochytrids are generally very lightly pigmented, they probably were under-reported in phytoplankton samples. Later analyses indicated that they comprise a significant portion of the phytoplankton community (29).

Prior to the implementation of the bio-rational collection/isolation program, there had never been any reports in the scientific literature that thraustochytrids were good lipid producers. There were two reports that thraustochytrids had long-chain n-3 FA in their lipids (30,31), but there were no reports of how much lipid these organisms could produce. In fact as late as 1992, Kendrick and Ratledge (32), reporting on their own data and that of Bajpai *et al.* (33), noted that *Thraustochytrium* had oil contents of only 10–15% of the biomass. They also noted that prospects for promoting high amounts of lipid in this microorganism appeared to be limited due to its lack of a key enzyme for lipid accumulation, ATP/citrate lyase. Contrary to this belief, using the bio-rational collection/isolation/screening procedures outlined previously, the authors were able to isolate thraustochytrid strains that could produce large amounts of lipid while having all of the other targeted characteristics for fermentation production and product use. Most significantly, these strains also had three important properties: (a) the isolated strains had very fast growth rates (6–9 doublings per day) compared with prior art strains of thraustochytrids (3–5 doublings per day); (b) high amounts of n-3 FA as a percentage of total FA were produced even at elevated temperatures (30°C); and (c) strains with enhanced lipid and DHA production could be grown at low salinities, and production at low salinities enhanced the production of DHA-rich lipids (Fig. 3.1).

Although there was a reduction in n-3 FA production at the very lowest salinities (Table 3.2), it was discovered that varying both the type of sodium salt and nitrogen salt used in the fermentation media could enhance the DHA concentration in the oil from 21 to 43% without a significant loss in biomass production at the very lowest salinities tested (Table 3.3). Since chloride is the component of marine media that contributes the most to metal corrosion, the authors worked to develop cultivation with a minimum content of both sodium and chloride (34). Chloride levels for fermentation media used in stainless steel fermentors should be less than 300 ppm to minimize stress cracking and corrosion. The results shown in Table 3.3 indicated that sodium sulfate could be an effective substitute for sodium chloride in the cultivation media, and eventually media were developed that resulted in excellent production at

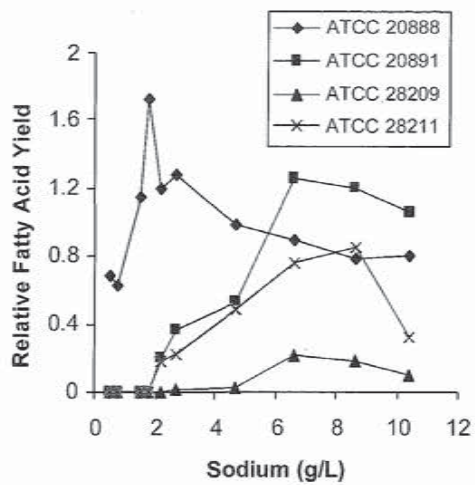


Fig. 3.1. Enhanced fatty acid yield of *Schizochytrium* sp. (ATCC 20888) (isolated by the bio-rational approach) at low sodium concentrations compared to fatty acid yield of previously known strains of thraustochytrids that had been isolated by other methods. Experimental conditions are outlined in Barclay (26).

chloride levels of less than 300 ppm (Table 3.4). Thus, by experimenting to solve the significant corrosion problem presented by growing marine organisms in stainless steel fermentors, the authors surprisingly found a way to also increase DHA production in strains of thraustochytrids. An additional surprise was that the sodium sulfate-based medium also appeared to further limit ectoplasmic net formation in *Schizochytrium*. One of the unique characteristics of thraustochytrids is that they produce ectoplasmic nets that tend to link the cells together. These nets can lead to clumped growth in liquid cultures, resulting in more slowly growing cells. The most rapidly growing strain isolated by the bio-rational method *Schizochytrium* sp. ATCC

TABLE 3.2
Production of *Schizochytrium* sp. (ATCC 20888) in Low Salinity Medium[a]

Na (g/L)	Cl (g/L)	Biomass (g/L)	Fatty Acids (% dry wt)	DHA[b] (% dry wt)	Final Glucose (g/L)
4.88	7.1	1.8 ± 0.6	35.4 ± 1.0	9.2 ± 0.5	0.0
3.90	3.9	5.7 ± 0.7	37.0 ± 0.7	10.0 ± 0.3	0.2
2.93	4.3	1.7 ± 0.4	43.0 ± 0.2	10.9 ± 0.1	0.2
1.95	2.9	1.7 ± 0.6	29.8 ± 0.7	8.4 ± 0.1	1.6
0.98	1.4	0.4 ± 0.6	10.6 ± 2.4	3.6 ± 1.0	4.3

[a]Experimental conditions are described in detail in Barclay (34).
[b]Abbreviation: Docosahexaenoic acid, DHA.

TABLE 3.3
Effect of Sodium Sulfate-Based Medium Compared with Sodium Chloride-Based Medium on Fatty Acid Content in *Schizochytrium* sp. (ATCC 20888)[a]

N source (g/L)	DHA[b] (% dry wt)	TFA (% dry wt)	Biomass (g/L)
Na salt = sodium chloride; N source = sodium glutamate			
3.0	5.6	11.2	1.74
2.5	5.5	10.8	1.71
2.0	5.5	11.0	1.65
1.5	7.1	20.3	1.39
Na salt = sodium chloride; N source = peptone			
3.0	7.4	21.0	1.34
2.5	8.8	27.4	1.21
2.0	6.3	28.9	1.18
1.5	10.4	42.1	1.16
Na salt = sodium sulfate; N source = sodium glutamate			
3.0	8.7	31.9	1.34
2.5	8.7	31.9	1.34
2.0	9.5	41.4	1.30
1.5	8.9	43.6	1.26

[a]Experimental conditions are described in detail in Barclay (34).
[b]Abbreviations: total fatty acid, TFA; see Table 3.2.

TABLE 3.4
Fermentation of *Schizochytrium* sp. (ATCC 20888) at Low Chloride Concentrations[a]

Chloride (mg/L)	Na 2.37 g/L Biomass Yield (mg/L)	Na 4.0 g/L Biomass Yield (mg/L)
0.1	198 ± 21	158 ± 48
0.7	545 ± 120	394 ± 151
15.1	975 ± 21	758 ± 163
30.1	1140 ± 99	930 ± 64
59.1	1713 ± 18	1650 ± 14
119.1	1863 ± 53	1663 ± 46
238.1	1913 ± 11	1643 ± 39

[a]Experimental conditions are described in detail in Barclay (34).

20888, however, was uniquely characterized in part by very limited ectoplasmic net production (Porter, D., personal communication). Surprisingly, ectoplasmic net production appeared to be further reduced when the cells were grown in a sodium sulfate-based medium, an additional benefit resulting from solving the corrosion problem.

Preliminary Toxicology Screen

In the late 1980s, little was known about the physiology and biochemistry of thraustochytrids as a group, and nothing had been published related to the presence or absence of toxins in this group of microalgae. As a result, a decision was made early in the technology development process to conduct a preliminary screen for toxins to ensure that this group of microalgae, which was becoming a focus of technology development and was novel from a biotechnological perspective, would be safe to utilize to produce a DHA-rich oil for use as a food ingredient. A literature survey on the phylum Heterokonta, to which the thraustochytrids belong, did not turn up any reports of toxins from thraustochytrids. For the Heterokonta there were reports of only two very remotely related types of microalgae that produced toxins: (a) the diatom *Pseudonitzschia* sp. (and possibly one species of *Chrysochromulina* sp.) that produced a neurotoxin called domoic acid (35) and (b) two species of *Prymnesium* (*P. parvum* and *P. patelliferum*) that produced toxic phospho-proteolipids called prymnesium toxins (36). To be safe, the authors analyzed *Schizochytrium* sp. biomass for the presence of domoic acid using the standard high-performance liquid chromatography (HPLC) method of Lawrence *et al.* (37), and no trace of this compound was detected. Additionally, the presence of pyrmnesium toxin was evaluated with the sensitive bioassay of Vanhaecke *et al.* (38) and Larsen *et al.* (39). The results indicated an absence of prymnesium toxin in *Schizochytrium*. Results from additional simple toxicity screens (monitoring growth and reproduction) using laying hens and two aquaculture organisms, *Artemia nauplii* and rotifers, that were fed *Schizochytrium* and *Thraustochytrium* biomass produced in 1-L fermentors, also suggested that there were no other toxins present in the thraustochytrid biomass. These preliminary safety results produced the confidence to move ahead and begin to scale up the production technology.

Fermentation Scale-Up

Scale-up of the fermentation technology was conducted in partnership with Kelco Biopolymers, San Diego, a division of Merck Pharmaceutical and later a division of Monsanto. This effort was combined with a classical strain improvement program conducted in-house. The progress of the scale-up work in terms of several DHA production parameters is summarized in Table 3.5. Initial productivity levels in the lab at the 14-L stage were approximately 22 g/L biomass in 48 h using 40 g/L glucose (26). Kelco engineers quickly doubled both DHA titer and productivity at the 14-L level. Using their data and a media optimization program that, in part, exploited alternative nitrogen sources, enhanced lipid production at low salinities, and especially at low chloride levels, the engineers were able to achieve cell concentrations of 65–70 g/L with a DHA titer of about 8 g/L, resulting in a doubling of the DHA productivity to 0.1 g/L/h.

Scale-up of the fermentation technology continued with a focus on fed batch approaches to supply both the carbon and nitrogen sources. During this time some data indicated that low dissolved oxygen levels greatly facilitated DHA production. This was contrary to the conventional biochemical wisdom that high dissolved oxygen levels facilitated DHA production, since molecular oxygen was considered necessary for the enzymatic process involving the formation of double (unsaturated) bonds in FA (40). Until recently, it was assumed that all PUFA were synthesized *via* variations of a single basic biochemical pathway (a soluble FA synthase system produced medi-

TABLE 3.5
Major Process Changes that Occurred During Scale-Up of the Fermentation Process and their Positive Effect on Various Parameters Related to DHA Production[a]

Major Scale-Up Process Changes	Cell conc. (dry wt g/L)	DHA Titer (g/L)	Lipid conc. (% dry wt)	DHA in Oil (% FAME)	DHA in Cells (% dry wt)	DHA Productivity (g/L/h)
Initial lab scale [low salinity, monosodium glutamate as nitrogen source; Barclay, (26)]	21	2	39	26	10	0.05
Scale-up of lab fermentation to 10,000 L scale	40	4	35–40	25–28	8–12	0.07
Low chloride + ammonium sulfate as nitrogen source	65–70	8	35–40	32	10–13	0.10
Fed batch + low DO + ammonium as N source + direct harvest on drum dryers; Bailey *et al.* (51)	170–210	40–50	50–73	35–45	22–25	0.45–0.55

[a]Abbreviations: dissolved oxygen, DO; fatty acid methyl esters, FAME; see Table 3.2.

um-chain saturated FA that were then modified by a series of membrane-associated elongation and oxygen-dependent desaturation reactions).

Evidence for an alternative pathway for PUFA synthesis came initially from studies of marine bacteria. Although many bacteria, such as *Escherichia coli,* were known to produce monounsaturated FA (primarily, *cis* vaccenic acid, 18:1 Δ11), it was generally thought that bacteria were incapable of PUFA synthesis. This opinion changed when DeLong and Yayanos reported detection of EPA and DHA in several strains of psychrophilic marine bacteria (41). A second major advance occurred when Yazawa (42) identified a segment of genomic DNA from an EPA-producing marine bacterium (*Shewanella* strain SCRC-2738) that, when transferred to *E. coli,* conferred EPA synthesis on those cells. Further analysis led to the identification of five genes that were necessary and sufficient for EPA accumulation in the transformed *E. coli* (42). Although the proteins encoded by the introduced *Shewanella* genes contained regions with homology to enzymes associated with FA synthesis, it was not clear how these specific activities contributed to EPA synthesis. Based on biochemical assays of extracts from *Shewanella,* Watanabe *et al.* (43) suggested that the EPA was the product of an aerobic desaturase and elongase pathway, presumably encoded by those five genes.

Metz *et al.* (44) provided a different interpretation. They noted that all of the enzymatic activities required for *de novo* synthesis of unsaturated FA could be identified in the *Shewanella* proteins. Some of the individual domains closely resembled those found in polyketide synthases while others more closely resembled those of FA synthesis systems. Polyketide synthase systems use the same basic reactions of FA but often do not complete the cycles resulting in highly derivatized end products that typically contain keto and/or hydroxyl groups as well as carbon-carbon double bonds in the *trans* configuration (45). Additionally, the linear products of polyketide synthase systems can be cyclized to produce very complex molecules, such as antibiotics, toxins, and many other secondary products (45,46). Domains with homology to known FA desaturases were notably lacking in the *Shewanella* proteins. Culturing *E. coli* expressing the *Shewanella* genes under anaerobic conditions provided additional evidence, eliminating the potential involvement of desaturases: EPA accumulation was not inhibited by the lack of oxygen. A rationale for the anaerobic incorporation of *cis* double bonds is provided by the presence of two domains with homology to the *E. coli* FabA protein. FabA is a dual function enzyme responsible for the formation of *cis* vaccenic acid *via* a dehydration reaction (forming a *trans* double bond in the carbon chain) in combination with a reversible *trans-cis* isomerization.

In combination, the data indicated that four of the *Shewanella* genes encoded subunits of an enzyme (a PUFA synthase) capable of *de novo* synthesis of EPA from the same small precursor molecule (malonyl-CoA) utilized in normal FA synthesis (47). The other gene, encoding a single domain protein, was identified as encoding an accessory enzyme (a phosphopantetheinyl transferase) that activates several domains of the complex (acyl carrier protein domains) by the addition of an essential co-factor. Genes encoding proteins with homology to the *Shewanella* EPA synthesis proteins have been found in several other PUFA-containing marine bacteria, including ones that accumulate DHA instead of EPA (48–50).

Metz *et al.* (44) and their collaborators at the Calgene Campus of Monsanto (Davis, California) discovered that *Schizochytrium* contained genes encoding proteins homologous to those associated with EPA synthesis in the marine bacterium *Shewanella* sp. strain SCRC-2738. The possibility that *Schizochytrium* possessed a similar pathway for DHA synthesis provided a rationale for the seemingly contradictory oxygen effects on DHA production. A key aspect of this novel PUFA biosynthetic system is that the *cis* double bonds are incorporated into the growing carbon chain *via* a dehydrase/isomerization mechanism rather than being inserted by oxygen-dependent desaturation reactions. *Schizochytrium* sp. (ATCC 20888) represented the first eukaryotic microorganism ever identified to contain a PUFA synthase system. The discovery that this microorganism did not require oxygen for long-chain unsaturated FA production synthesis assisted in efforts to begin to grow this organism at very high densities.

Final scale-up of the fermentation involved incorporation of four key components: (a) low chloride media; (b) fed batch supply of the carbon and nitrogen sources; (c) use of low dissolved oxygen levels to induce DHA formation; and (d) harvesting of the resulting biomass by recovering the fermentation broth directly on drum driers (avoiding use of centrifugation). This process was outlined by Bailey *et al.* (51). Using this process, biomass yields of greater than 200 g/L could be achieved with DHA concentrations in the dried biomass of greater than 20%. This resulted in DHA productivities >0.55 g/L/h.

During the fermentation scale-up process from laboratory-scale 14-L fermentors to 150,000-L commercial fermentors, the following key improvements in DHA pro-

duction parameters were achieved: (a) Final cell dry weight concentrations improved from 21 to 170–210 g/L; (b) DHA concentrations in the resulting oil increased from 26 to 35–45% of total FA; (c) DHA concentration as a percentage of the dry weight of the cells increased from 10 to 22–25%; and (d) DHA productivity increased from 0.05 to 0.45–0.55 g/L/h, a 10-fold increase. These cell concentrations and DHA productivities are the highest ever reported from a microbial production technology to produce highly unsaturated FA. The conventional methods used to extract and refine the resulting DHA-rich oil from this process have been outlined by Zeller *et al.* (52). The FA profile of the refined DHA-rich oil is illustrated in Table 3.6.

Safety of the Biomass and Extracted DHA-Rich Oil

After scale-up of a representative and reproducible fermentation process was completed, a series of standard toxicology studies was conducted to demonstrate the safety of the whole cell, DHA-rich biomass and extracted oil. The strategy behind the selection of these studies and the results are discussed in greater detail in Chapter 10. These studies included: (a) mutagenicity studies (*in vitro* Ames and mammalian cell line, *in vitro* human peripheral blood lymphocytes, and *in vivo* mouse micronucleus) (53); (b)

TABLE 3.6
Example Fatty Acid Profile of the Refined Oil Produced from the *Schizochytrium* Fermentation Process[a]

Fatty Acid	Fatty Acid Content (FAME, mg/g)	(% TFA)
12:0	2	0.2
14:0	71	7.6
14:1	1	0.1
15:0	4	0.4
16:0	205	22.1
16:1	4	0.4
18:0	5	0.5
18:1	7	0.8
18:2	5	0.5
18:3(n-6)	3	0.3
18:4	3	0.3
20:0	1	0.1
20:4(n-6)	9	1.0
20:4(n-3)	9	1.0
20:5(n-3)	21	2.3
22:0	1	0.1
22:5(n-6)	154	16.6
22:6(n-3)	380	40.9
24:0	2	0.2
24:1	2	0.2
Others	40	4.3

[a]Abbreviations: see Tables 3.2 and 3.3.

a 13-wk sub-chronic rat feeding study (54); (c) developmental toxicity evaluation in rats and rabbits (55); and (d) a single-generation rat reproduction study (56). An acute gavage trial (extracted oil) in mice was also conducted, along with target animal safety studies in laying hens (57), broiler chickens, and swine (58). The no observable adverse effect level for all safety tests was at the highest dietary level of the oil tested in each study. Based on the amount of DHA-rich oil fed to the test animals these levels were: (a) one generation rat reproduction study, 8800 mg oil/kg/d; (b) rat teratology study, 8400 mg oil/kg/d; (c) rabbit teratology study, 720 mg oil/kg/d; (d) 13-wk rat feeding study, 1600 mg oil/kg/d; (e) laying hen target animal safety test (16 wk), 1600 mg oil/kg/d; and (f) broiler target animal safety test (7 wk), 950 mg oil/kg/d.

The results of these safety studies allowed a Generally Recognized as Safe (GRAS) determination to be made by a committee of food safety experts, qualified by scientific training and experience, on the whole cell biomass for use in poultry feed and for use of the extracted DHA-rich oil in foods. Subsequently, in 2004, the U.S. Food and Drug Administration did not object to the GRAS determination on the safe use of the DHA oil from *Schizochytrium* in foods. Novel Foods approval was also awarded for the use of the oil in foods in Australia and New Zealand in 2002 and in Europe in 2003.

Products

The DHA-rich biomass resulting from this fermentation process has been evaluated for use in enrichment applications in aquaculture (59) and is now used throughout the world in aquaculture feeds for enrichment applications and as a nutritional ingredient in feed for larval fish and shrimp (see www.aquafauna.com/Diets&Feeds.htm). It has also been used in poultry feed to produce DHA-enriched eggs (60) and poultry meat, and in Europe to produce DHA-enriched milk from dairy cows. Research has indicated that the DHA-rich biomass can also be fed to swine to enrich the resulting meat with DHA without any compromise in meat characteristics, sensory attributes, or tenderness (61–63). The extracted DHA-rich oil is sold in capsules as a nutritional supplement and has been used commercially as an ingredient in nutritional bars and soy milk drinks. It is currently being evaluated as an ingredient in several dairy products including yogurts, spreads, margarines, and cheeses.

Conclusion

A bio-rational approach was used to develop a production technology for long-chain n-3 FA. The innovative collection/isolation/screening program resulted in the isolation of strains of a unique group of microorganisms, thraustochytrids, with significant biotechnological potential. The best strains contained all of the targeted production characteristics to enable the inexpensive production of long-chain n-3 FA. These strains were unicellular and small (<25 μm), exhibited fast growth, high n-3 FA content even at high temperatures, and excellent n-3 FA-rich lipid production at low

salinities. The strains also exhibited two completely unexpected characteristics: (a) enhanced long-chain n-3 FA production in low-salinity and especially low-chloride production media; and (b) enhanced production of long-chain n-3 FA under very low dissolved oxygen concentrations. Scale-up of this technology, using a strain of *Schizochytrium* sp., resulted in a fermentation technology with the highest cell concentrations (about 200 g/L), highest unsaturated lipid productivities (about 0.55 g/L/h), and the highest cellular concentrations of DHA (about 22% dry weight) ever reported for a microbial production technology. In addition to the unique combination of fermentation characteristics, another key to these exceptional results was the unique genetic system that these strains possessed to produce long-chain polyunsaturated FA. These strains represented the first eukaryotic microbes ever discovered to utilize a polyketide-like PUFA synthase system to produce long-chain polyunsaturated FA.

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Chapter 4

Searching for PUFA-Rich Microalgae

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Introduction

Several very long-chain polyunsaturated fatty acids (VLCPUFA) of C_{20}-C_{22} carbon atoms are of value for various nutritional and pharmaceutical purposes. Some of these polyunsaturated fatty acids (PUFA) are precursors to different families of prostaglandins and leukotrienes. Arachidonic acid (AA, 20:4n-6, ARA) and docosahexaenoic acid (DHA, 22:6n-3), which are the major PUFA of brain membrane phospholipids, are necessary for visual acuity and improved cognitive development of infants (1,2). Newborns obtain most of these PUFA from breast milk (3), and it was thus suggested that the diet of preterm infants who are not breast-fed should be supplemented with these PUFA (4,5). Indeed, various health authorities now recommend the incorporation of both AA and DHA into baby formulae (6) and the FDA has recently approved their combined use.

VLCPUFA of the n-3 family are abundant in microalgae. For example, *Porphyridium cruentum* (7), *Nannochloropsis* sp. (8,9), *Phaeodactylum tricornutum* (10,11), and *Monodus subterraneus* (12) were studied for their potential to produce eicosapentaenoic acid (EPA, 20:5n-3). Likewise, *Crypthecodinium cohnii* (13), and *Chroomonas salina* (14) contain DHA. However, very long-chain n-6 PUFA are relatively rare, and high contents of 20:3n-6 are not found in any organism unless it has undergone genetic manipulation (see Chapter 2). AA is almost nonexistent in the lipids of fresh water algae and, in most marine species it does not account for more than a few percent of total fatty acids (TFA) [Table 4.1 (15)].

Microalgae can be induced to accumulate large amounts of oil, mostly in the form of triacylglycerols (TAG); however, very little of the accumulated fatty acids (FA) are PUFA (23). In order for microalgae to be used as an economical source of VLCPUFA, strains that can accumulate these PUFA in their TAG must first be found before conditions leading to increased and rapid accumulation can be identified. For the last two decades the authors have studied the composition, production, accumulation, and role of PUFA-rich TAG in microalgae.

Occurrence of PUFA-Rich TAG in Microalgae

Many works describe the production of DHA (24), EPA (11,23,25,26), and γ-linolenic acid (GLA, 18:3n-6) (27) from microalgae. However, very few have studied the pro-

TABLE 4.1
Major Fatty Acid Composition of Algae Relatively Rich in Arachidonic Acid (AA)[a]

	Major Fatty Acids (% of Total)									
Species	16:0	16:1	18:1	18:2	18:3 n-6	18:4 n-3	20:4 n-6	20:5 n-3	22:6 n-3	Ref.
Bacillariophyceae										
Thalassiosira pseudonana	10	29	—	1	—	—	14	15	—	17
Chlorophyceae										
Parietochloris incisa	10	2	16	17	1	—	43	1	—	16
Dinophyceae										
Amphidinium carteri	12	1	2	1	3	19	20	—	24	17
Phaeophyceae										
Desmarestia acculeata	12	2	7	6	10	16	19	19	—	18
Dictyopteris membranacea	20	1	14	14	11	11	11	9	—	19
Ectocarpus fasciculatus	17	—	13	4	15	23	11	13	—	20
Prasinophyceae										
Ochromonas danica	4	—	7	26	12	7	8	—	—	21
Rhodophyceae										
Gracilaria confervoides	18	3	16	2	—	1	46	—	—	18
Phycodrys sinuosa	22	5	5	1	1	—	44	2	—	18
Porphyridium cruentum	34	1	2	12	—	—	40	7	—	22

[a]Source: Reference 16.

duction of AA. Until recently, the only microalga reported to produce AA in significant quantities was the unicellular rhodophyte *Porphyridium cruentum* (22). An unfortunate choice of chromatographic conditions led Ahern *et al.* (28) to erroneously suggest that *P. cruentum* contains AA only as its C_{20} PUFA, and that it could accumulate AA up to 8% of its dry weight. Over 10 years ago, Cohen (22) showed that under nitrogen starvation, *P. cruentum* can be induced to accumulate only up to 2.5% AA (dry wt) and 41% of TFA. The latter was, until recently, the highest content of AA found in any plant source. The predominant reason for the low content of AA is that it acts either as an intermediate in the biosynthesis of EPA or, when it does occur as a major PUFA in algae, the FA content is rather low; in red macroalgae, AA can amount to 60% of TFA (Table 4.1) but its dry weight content will rarely exceed 1% (23). Some fungi, however, especially of the genus *Mortierella*, were shown to accumulate AA up to 60% of their FA (29; see also Chapter 2).

Accumulation of PUFA in Microalgae

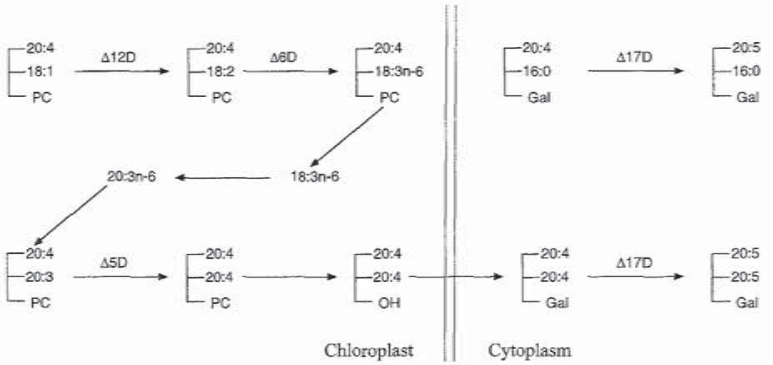
Oil accumulation in microalgae is a biphasic process. Rapid cell division will continue as long as growth conditions are not limiting. In the lipogenic phase, excess carbon and a growth factor limitation, most often nitrogen, can result in the accumulation of lipids (30). Imposing nitrogen limitation when light is in excess results in cessation of growth. Since photosynthetic fixation of carbon continues, the cellular ratio of C/N increases (31) and carbon can be channeled into production of non-nitrogenous

reserve materials, such as TAG, that serve as a sink for photosynthetically fixed carbon. Many algae have demonstrated TAG accumulation of up to 70% of dry weight (32,33). However, algal TAG are generally characterized by the presence of saturated and monounsaturated FA (34–36). In *P. cruentum*, N-starvation resulted in an enhanced accumulation of TAG from 21 to 61% of total lipids (22). Surprisingly, the proportion of AA in TAG increased from 20 to 31% (7). According to the prevailing understanding, TAG are accumulated in algae predominantly as an energy reserve (18,37). For this purpose, saturated acyl moieties should be preferred, since they require less energy to produce than PUFA and provide more energy upon oxidation. Energetically, therefore, there is very little sense in producing and accumulating PUFA-rich TAG. The unique FA composition of the TAG of *P. cruentum* led us to look for alternative roles of TAG in phototrophic microalgae.

Biosynthesis of PUFA in *P. cruentum*

In order to understand the roles of PUFA in *P. cruentum*, we studied the biosynthetic pathways leading to the production of these PUFA in depth. The tools used include assimilation of external of FA (38), desaturase inhibitors (39), and time-course studies following the incorporation of label using radioactive FA (40). Based on the results of these experiments, it was concluded that in the predominant pathway oleate is incorporated into phosphatidyl choline (PC) and stepwise desaturated to 18:3n-6; this is then elongated to 20:3n-6. The latter is re-incorporated into PC and further desaturated to AA (Scheme 4.1). AA is imported to the chloroplast where it can be further desaturated to EPA, mostly as a component of the galactolipid monogalactosyldiacylglycerol (MGDG).

In higher plants and many green algae, chloroplastic lipids are divided into two types. Prokaryotic lipids are characterized by the presence of C_{16} FA in the *sn*-2 posi-



Scheme 4.1. Outline of the pathways leading to eicosapentaenoic acid (EPA) biosynthesis in *Porphyridium cruentum*.

tion and either C_{16} or C_{18} FA at the *sn*-1 position of their glycerol skeletons, 16/16 and 18/16, respectively. (Pairs of numbers that represent the FA separated by a solidus designate the components in the *sn*-1 and *sn*-2 positions, respectively.) Eukaryotic lipids contain C_{18} FA at both positions (18/18). The C_{18} acyl moieties of the prokaryotic lipids are entirely produced in the chloroplast while one or two of the desaturation steps of the eukaryotic pathway occur using extra-chloroplastic lipids (41). The authors have suggested that the molecular species of the chloroplastic lipids of VLCPUFA-containing microalgae can be similarly divided into prokaryotic-like (20/16) and eukaryotic-like (20/20). As was determined later, these molecular species resemble those of higher plants in structure but differ in their biosynthetic origin. In every microalgal strain studied, the lipid-linked biosynthesis of VLCPUFA is exclusively extra-chloroplastic up to the stage of C_{20} PUFA (42).

The Molecular Species Composition of the Galactolipids of *P. cruentum* Is Modified by Temperature

Some of the earliest reports studying *P. cruentum* showed that the FA composition varied according to its environmental conditions (35,43,44). Under optimal conditions, the major PUFA is EPA. However, any change in the nutritional or environmental conditions decreased EPA and increased its precursor, AA. A shift from n-3 to n-6 PUFA in chloroplastic lipids was reported to occur in many microalgae upon transition from the logarithmic to the stationary phase. However, the magnitude of the effect was much more intense in *P. cruentum*. Klyachko-Gurvich *et al.* (45) suggested that n-3 PUFA are necessary for the maintenance of photosystem I and that accumulation of the n-6 precursors in the stationary phase enables the organism to rapidly produce n-3 PUFA once growth conditions are resumed. In contrast to *P. cruentum*, the last n-3 desaturation stage is also extra-chloroplastic in most EPA-rich microalgae studied. We speculated that these differences originate from the disparity in the ecological niches to which these organisms were adapted.

In one of our earliest studies of *P. cruentum* (7), we showed that, as expected, the ratio of n-3/n-6 PUFA in monogalactosyldiacylglycerol (MGDG), the main depot of PUFA in the alga, increased with decreasing temperature. However, the ratio of PUFA to 16:0 increased. This clearly suggested a change in the distribution of the molecular species. Indeed, the proportion of the eukaryotic-like molecular species of MGDG in *P. cruentum* is inversely proportional to the growth temperature (Table 4.2). At 30°C, 21% of the molecular species were eukaryotic-like, compared to 29% at 25°C and 58% at 20°C (46). However, the molecular species composition of digalactosyldiacyglycerol (DGDG), which is almost exclusively prokaryotic-like, was hardly affected. These findings were surprising, since we were not aware of any reports concerning temperature-related changes in the ratio of eukaryotic to prokaryotic molecular species in either higher or lower plants. This led to the suggestion that the presence of two types of molecular species may provide a useful mechanism for regulating the physical properties of chloroplast membranes in response to changes in

TABLE 4.2
Molecular Species Composition of Monogalactosyldiacylglycerol (MGDG) and Digalactosyldiacylglycerol (DGDG) of *P. cruentum* Cultivated at Different Temperatures

		Molecular Species (% of Total)		
		Temperature (°C)		
Lipid	Molecular Species	20	25	30
MGDG	20:5/20:5	46	26	12
	20:4/20:5	11	2	7
	20:4/20:4	1	tr	2
Total Eukaryotic-like		58	29	21
	20:5/16:0	20	54	54
	20:4/16:0	2	2	16
	18:2/16:0	20	15	10
Total Prokaryotic-like		42	71	80
DGDG	20:5/20:5	2	1	tr
	20:4/20:5	tr	—	tr
	20:4/20:4	—	—	—
Total Eukaryotic-like		2	1	1
DGDG	20:5/16:0	87	91	74
	20:4/16:0	2	2	14
	18:2/16:0	8	7	11
Total Prokaryotic-like		97	99	99

Abbreviation: tr, trace (<0.1%).
Source: Reference 46.

environmental conditions. In higher plants, the difference between the prokaryotic (18:3/16:3) and eukaryotic (18:3/18:3) molecular species end products of MGDG is quite subtle and is expressed in a slightly shorter acyl group at the *sn*-2 position of the prokaryotic species. Therefore, shifts between prokaryotic and eukaryotic molecular species are of lesser consequence. However, in *P. cruentum* the major eukaryotic-like species (20:5/20:5) of MGDG contains five more double bonds and four more carbon atoms than the prokaryotic-like molecular species (mainly 20:5/16:0).

Selection of Chill-Sensitive Mutants of *P. cruentum* Reveals a Novel Role for TAG

To test the hypothesis concerning the significance of eukaryotic-like MGDG molecular species, we looked for cold-sensitive mutants deficient in EPA production, especially in the eukaryotic pathway. A search for cold-sensitive mutants of *P. cruentum* was launched on the assumption that EPA in this microalga is important for photosynthesis and is similar to 18:3n-3 in higher plants and green algae (7). By comparing the growth of putative mutants on agar plates at 15 and 25°C with that of the wild type,

the authors succeeded in selecting a series of cold-sensitive lines. In one of these, the HZ3 mutant, growth was severely inhibited at 15°C, but not at 25°C.

The lipid composition of the HZ3 mutant showed a five-fold increase in the proportion of TAG compared with the wild type and a corresponding decrease in that of PC (Table 4.3) (47). The proportion of EPA decreased to 27%, compared to 41% in the wild type. The most affected lipid was MGDG in which the proportion of EPA decreased from 63 to 42%. However, other chloroplastic lipids were not affected. This difference between the lipids is clearly due to MGDG being mostly eukaryotic-like, while other chloroplastic lipids are almost entirely prokaryotic-like. Indeed, the proportion of the dominant eukaryotic-like molecular species of MGDG, 20:5/20:5, decreased but that of the prokaryotic-like species 18:2/16:0, 20:4/16:0, and 20:5/16:0 increased. However, the DGDG molecular species composition was not significantly changed, indicating that the mutation had affected only the production of eukaryotic-like molecular species.

Following pulses of ^{14}C-labeled FA, wild-type TAG rapidly lost its label in favor of the chloroplastic lipids; however, labeled FA within the TAG of the mutant remained relatively stable. In contrast, PC of both wild type and mutant turned over their label rapidly. As expected, mutant eukaryotic-like MGDG showed lesser and slower labeling than that of the wild type, further supporting a deficiency in the eukaryotic pathway (40).

Recovery from Nitrogen Starvation

Under nitrogen starvation, much of the acyl flux of *P. cruentum* is diverted from the production of chloroplast lipids (predominantly eukaryotic-like MGDG) to the accumulation of TAG in oil bodies (22). When nitrogen is replenished, new chloroplast lipids are produced, while oil bodies decrease (48). After nitrogen starvation for 2 d, wild type and HZ3 mutant cultures of *P. cruentum* were labeled for 24 h with [1-^{14}C] AA. The incorporated label, as well its distribution between cytoplasmic and chloroplastic lipids, was similar in both cultures. Following nitrogen replenishment, a massive transfer of label from TAG to eukaryotic-like MGDG, in which the label increased four-fold, was observed in the wild type. Wild-type TAG lost 90% of its label, compared to only 59% in the mutant (47). Correspondingly, labeling of all the eukaryotic-like species of MGDG in the mutant was about one-half of that of the wild type. At present, the mechanism by which TAG re-utilization occurs remains unknown. Nonetheless, several possibilities could be considered: a transacylation of monoacylglycerol (MAG) by TAG to produce two molecules of diacylglycerols (DAG) (49), a transacylation of two molecules of DAG, and a lipase activity that hydrolyzes TAG to DAG.

TABLE 4.3
Lipid and Major Fatty Acid Composition of Wild Type (WT) and HZ3 Mutant of *P. cruentum*

			Major Fatty Acid Composition (% of Total)			
Strain	Lipid Class	% of Total	16:0	18:2n-6	20:4n-6	20:5n-3
WT	Total lipids	100	26	5	18	41
HZ3	Total lipids	100	31	4	23	27
WT	MGDG	40	26	4	6	63
HZ3		33	33	7	14	42
WT	DGDG	22	46	5	2	45
HZ3		22	46	5	6	41
WT	PC	10	27	4	57	5
HZ3		8	18	2	68	3
WT	TAG	2	21	21	33	17
HZ3		10	22	24	40	5

Cultures were cultivated at 25°C under a regime of daily dilution.
Abbreviations: Phosphatidylcholine, PC; triacylglycerol, TAG. For other abbreviations see Table 4.2.
Source: Reference 23.

In higher plant leaf lipids, TAG share a common DAG pool with phospholipids, primarily with PC. However, the conversion of DAG to TAG is generally considered to be unidirectional (50). The findings of Khozin *et al.* (40) clearly show that, in addition to PC, there is a significant contribution of TAG to the synthesis of chloroplastic lipids of *P. cruentum*. The ability of this organism to utilize its TAG may explain the unique richness in AA and EPA in these lipids.

While most algae grow in large water bodies in which temperature only changes slowly, *P. cruentum* is found in shallow marshes and wet sands in which the temperature can vary rapidly. The increase in the proportion of EPA in MGDG, especially in the eukaryotic-like component of MGDG (20:5/20:5), observed at low temperatures, may reflect the organism's mode of coping with stress inflicted by sudden temperature changes. Arguably, TAG could be used as a buffering capacity for AA and EPA-containing DAG; these could be relatively rapidly mobilized to produce eukaryotic-like molecular species of MGDG. The HZ3 mutant is deficient in this pathway and compensation by enhanced production of prokaryotic-like species is inherently limited. However, the ability of the mutant to import DAG moieties from PC into the chloroplast to produce eukaryotic molecular species of MGDG appeared to be normal.

PUFA-Rich TAG Can Be Used as a Reservoir of PUFA for the Modification of Chloroplastic Lipids

The increase in the degree of unsaturation of membrane FA upon temperature reduction is a universal phenomenon (51). Therefore, adaptation to sudden decreases in temperature must include a mechanism to rapidly enhance the PUFA content of the membranes. However, the rate of biochemical processes slows down at lower temperatures. Even at room temperature, radiolabeling of *P. cruentum* with acetate has shown that labeled EPA appeared in the chloroplast more than 10 h after the pulse (40), suggesting that algae that are exposed to rapid changes of temperature would have difficulty increasing their chloroplastic PUFA content at low temperatures by *de novo* synthesis. The protec-

tive role of PUFA against the damaging effect of high intensity light and UV radiation, especially at low temperatures, may also be important for the ability of the organism to survive and adapt in extreme environments (52). The capability to store PUFA in TAG would allow the organism to adapt swiftly to the rapidly changing environment. Since high levels of free FA would be toxic to the cell, these must be stored in the form of lipids. Polar lipids are membrane components and consequently, accumulation of these lipids is intrinsically limited; this leaves neutral lipids, predominantly TAG, as the only viable option. We have thus hypothesized that in algae whose natural habitat is characterized by rapid changes in environmental conditions, such as alpine environments, VLCPUFA-rich TAG might be involved in a buffering capacity for PUFA.

Isolation and Characterization of *Parietochloris incisa*

In order to test this hypothesis, several algal species were collected from the soil of Mt. Tateyama, Japan (Boussibou, S., Richmond, A., and Vunshak, A., unpublished work). This ecological niche is characterized by a wide range of temperatures, from freezing to over 20°C, and light intensity varying from normal to very high, due to reflection from snow. Screening of the strains resulted in the identification of the chlorophyte (trebouxiophyceae) *P. incisa* (53) as the richest known plant source of AA (16). This strain is also, to the best of our knowledge, the first reported alga capable of accumulating large quantities of TAG that are particularly rich in any PUFA. AA is the major FA of *P. incisa*, comprising 34% of TFA in the logarithmic phase and 43% in the stationary phase (Table 4.4). Other major FA are 16:0, 18:1, and 18:2. Among the minor FA, there are n-6 PUFA, such as 18:3n-6, and 20:3n-6, as well as PUFA of the n-3 family, 16:3n-3, 18:3n-3, and 20:5n-3. Even in logarithmic cultures, TAG was the major lipid class and accounted for 43% of TFA (Table 4.4). In the stationary phase, its proportion increased even further to 77%. In contrast to most algae whose TAG are made of mainly saturated and monounsaturated FA, the TAG of *P. incisa* are the major lipid class in which AA is deposited, and reach up to 47% in the stationary phase. Other than AA, TAG also contain 16:0, 18:1, and 18:2. Due to the sharp increase in TAG accumulation in the stationary phase (Table 4.4), the share of cellular AA that was deposited in TAG increased from 60% in the logarithmic phase to 90% in the stationary phase. With the exception of AA, the FA composition of the chloroplastic lipids was not too different from that of typical green algae, such as *Chlorella* (54), consisting mostly of C_{16} and C_{18} PUFA.

The lipid composition of *P. incisa* is also unusual. The simultaneous presence of diacylglyceroltrimethylhomoserine (DGTS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) is not very common. DGTS is abundant in many species of green algae, such as *Dunaliella salina, Chlamydodmonas reinhardtii,* and *Volvox carteri;* it appears to be located in non-plastidial membranes (15,37). DGTS resembles PC in some aspects and generally occurs when PC is either low or absent (55). The co-existence of these three lipids also occurs in the EPA-producing eustigmatophytes, *Nannochloropsis* sp. (56), and *M. subterraneus* (57). Generally DGTS appears

TABLE 4.4
Fatty Acid Composition of Major Lipids of *P. incisa* in the Logarithmic (L) and Stationary (S) Phases

			Fatty Acid Composition (% TFA)												
Lipid Class		Lipid Dist.[a]	16:0 n-11	16:1 n-6	16:2 n-3	16:3	18:0 n-9	18:1 n-7	18:1 n-6	18:2 n-6	18:3 n-3	18:3 n-6	20:3 n-6	20:4 n-3	20:5
Biomass	L		13.9	4.7	1.7	4.0	1.7	6.7	5.1	13.2	1.5	10.3	1.2	33.6	1.7
	S		10.1	1.8	1.3	0.9	2.5	12.2	4.2	17.2	0.8	2.0	1.0	42.5	0.7
MGDG	L	22.1	1.9	1.0	8.5	20.5	0.4	3.6	0.8	14.5	0.8	31.9	0.3	13.9	1.1
	S	4.9	3.4	1.2	20.8	11.0	0.5	4.3	0.0	31.4	0.7	18.5	0.2	6.1	0.6
DGDG	L	14.2	16.0	1.1	1.4	1.7	1.9	5.8	4.0	26.0	1.5	18.6	0.7	18.1	1.4
	S	6.8	34.0	0.5	0.2	0.6	4.5	6.9	3.0	31.0	2.4	2.9	0.4	7.6	0.6
PC	L	5.7	29.5	2.7	0.2	0.5	4.2	11.8	2.0	16.0	4.5	2.3	1.9	21.0	1.8
	S	0.7	31.2	1.4	—	—	5.5	8.9	15.4	21.1	2.6	1.4	0.7	9.6	—
DGTS[b]	L	4.0	47.1	1.8	0.3	0.5	7.4	2.0	7.4	9.4	3.6	1.9	0.9	15.2	0.8
	S	4.6	30.0	0.4	2.6	0.6	3.9	5.4	2.8	34.2	3.3	3.5	0.4	10.0	0.6
PE[b]	L	2.9	11.1	1.4	0.2	1.3	3.9	4.2	18.2	4.8	1.2	3.0	4.1	43.2	1.7
	S	0.6	19.8	4.5	0.3	4.8	7.1	4.3	24.7	11.2	1.5	1.8	2.0	14.3	—
TAG	L	42.9	13.3	0.5	tr	0.4	3.7	15.3	6.8	10.4	1.1	1.0	1.5	43.0	1.0
	S	77.1	8.4	0.4	tr	tr	3.1	18.0	4.0	14.1	0.7	0.4	1.1	47.1	0.7

[a]Lipid distribution (% TFA); — undetected.
[b]Abbreviation: Phosphatidylethanolamine, PE. diacylglyceroltrimethylhomoserine, DGTS. For other abbreviations, see Tables 4.2 and 4.3.
Source: Reference 16.

together with either PC or PE, but not both. For example, *Chlamydomonas reinhardtii* (58) contains PE and DGTS, while PC and DGTS are found in *D. parva* (59) and *D. salina* (60). We have speculated that the co-occurrence of these lipids in *P. incisa* is related to the production of AA.

Induced Accumulation of AA in *P. incisa*

To induce N-starvation, cultures of *P. incisa* at the early stationary phase were resuspended and maintained in a nitrogen-free medium (61). Electron microscopy revealed that TAG was accumulated in large oil globules (Fig. 4.1). After 14 days, the FA content of the N-free culture increased from 17 to 36% (dry wt), compared to 25% in the control (Table 4.5). The FA composition demonstrated a sharp increase in the proportion of AA from 40 to 59% of TFA, compared to only 46% in the control. The proportion of AA increased in both glycolipids and phospholipids, but especially in neutral lipids, in which it increased to 64%, compared to 51% in the control. Correspondingly, the dry weight content of AA increased from 7 to 21% in the N-free culture and to 11% in the control culture. Neutral lipids, mostly TAG, comprised 87% of TFA, compared to 62% in the control (Table 4.6). Under N-starvation, over 90% of cellular AA was in the TAG. The enhancement in the content of AA under nitrogen starvation was the result of both the increase in the proportion of TAG and the increase of AA in TAG.

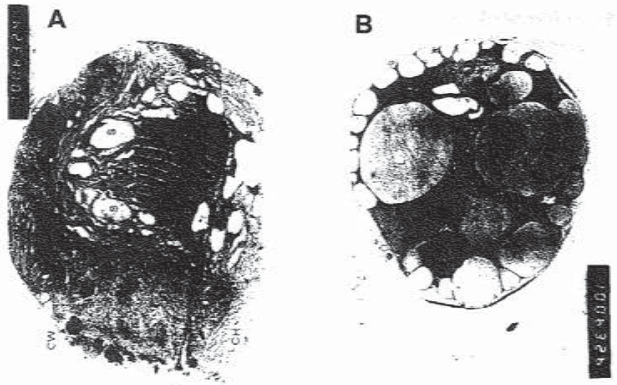


Fig. 4.1. Electron microscopy of *Parietochloris incisa*. (A) Cells cultivated under optimal conditions. (B) Cells harvested after 14 days of N-starvation.

The major molecular species of TAG was triarachidonylglycerol, whose share of total TAG was as high as 40%, compared to only 21% in the control (Table 4.7). The total share of three other molecular species containing two arachidonyl moieties (as well as 18:2, 18:1, or 16:0) amounted to 54%, whereas molecular species containing only one arachidonyl moiety almost disappeared.

The accumulation of FA following nitrogen starvation is driven by the increase in the C/N ratio in the medium. Increasing the availability of carbon could enhance FA accumulation even further. We have thus studied the effect of the addition of acetate to the medium of N-starved cultures of *P. incisa* (Cohen Z. and Khozin-Goldberg I., unpublished work). While there was some effect on the FA content, the FA composition was adversely affected. The major increase was noted in the proportion of 18:1, which increased up to 40%, at the expense of AA. Consequently, the AA content was lower. Labeling experiments revealed that excess of acetate was used not only for the

TABLE 4.5
Effect of Nitrogen Starvation on the Composition and Content of Fatty Acids in *P. incisa*[a]

		Content (% dry wt)		Major Fatty Acid Composition (% of Total)							
Culture	Time (d)	TFA	AA	16:0	18:0	18:1 n-9	18:1 n-7	18:2 n-6	18:3 n-6	18:3 n-3	20:4 n-6
Control	0	16.5	6.6	11	2	11	5	17	1	4	40
Control	14	24.7	11.4	9	2	14	4	17	1	2	46
-N	14	35.8	21.1	9	2	9	6	9	1	1	59

[a]Cultures were resuspended in N-Free (-N) or control medium and cultivated in columns for another 14 d.
Source: Reference 61.

TABLE 4.6
Major Lipid Distribution of N-Starved Cultures of *P. incisa*[a]

			Major Fatty Acid Composition (% TFA)							
Culture	Lipid Fraction	Lipid Share (% TFA)	16:0	18:0	18:1 n-9	18:1 n-7	18:2 n-6	18:3 n-6	18:3 n-3	20:4 n-6
-N	NL	87	8	2	9	4	9	tr	tr	64
Control	NL	62	9	1	15	6	10	tr	2	51
-N	GL	10	12	2	5	4	11	1	12	30
Control	GL	19	9	1	5	3	21	tr	15	21
-N	PL	3	26	3	3	14	13	3	3	29
Control	PL	19	17	1	6	11	19	1	7	25

[a]For cultivation details see Table 4.5.
Abbreviations: Galactolipids, GL; neutral lipids, NL; phospholipids, PL.
Source: Reference 61.

TABLE 4.7
Major Molecular Species Composition of TAG in Stationary (Control) and Nitrogen Starved (-N) Cultures of *P. incisa*[a]

	Growth Medium	
Molecular Species[b] (% of Total)	Control	-N
20:4/20:4/20:4	21	40
20:4/20:4/18:2	17	16
20:4/20:4/18:1	18	20
20:4/20:4/16:0	11	18
20:4/18:2/16:0	15	2
20:4/18:2/18:1	11	—
20:4/18:1/18:1	2	1
20:4/18:1/18:0	3	—

[a]TAG were separated using reverse phase high-performance liquid chromatography. Peaks identified by Ultraviolet detection were collected, transmethylated, and analyzed by gas chromatography. Relative composition was estimated by integrating peak areas obtained using an evaporative light-scattering detector without calibration.
[b]Positional distribution was not determined; the values are given without assignment of any position of the TAG.
Source: Reference 61.

de novo FA synthesis, but also by the C_{16} elongase that converts 16:1 to 18:1. Apparently, the levels of the obtained oleate were too high and it competed with AA for incorporation into TAG instead of being incorporated into phospholipids for further desaturation.

Effect of Cell Density on AA Content

Generally, algal cultures produce more TAG when kept at a low biomass concentration and therefore, relatively high light per cell (33). This is the case for most PUFA-producing algae, such as the eustigmatophytes *Nannochloropsis* (26) and *M. subterra-*

neus (12), the diatom *P. tricornutum*, and many other EPA-rich species (62), all of which are rich in EPA. In contrast, in *P. incisa* FA, production (mostly as TAG) was enhanced under high biomass concentration in both nitrogen replete and especially in nitrogen-depleted cultures (Fig. 4.2). Similar results were obtained in another AA producer, *P. cruentum* (7). Possibly, low light per cell signals the end of the exponential phase and the beginning of the stationary phase. Thus, algae that accumulate TAG as an energy source would generally enhance TAG biosynthesis under high light conditions as a means of converting excess light to reserve energy. The FA composition of these TAG would be characterized by the predominance of saturated and monounsaturated FA. However, algae that produce PUFA-rich TAG, such as *P. cruentum* and *P. incisa*, can also utilize TAG as a reservoir of building blocks for the construction of chloroplastic lipids (63). Such TAG would be primarily accumulated under conditions resulting in slow growth, for example high biomass concentration or low light.

Biosynthesis of AA in *P. incisa*

The biosynthesis of AA in *P. incisa* was mainly revealed based on labeling experiments (64). Acetate labeling established that the biosynthetic sequence was similar to that of *P. cruentum* at the FA level. However, its apparent participation at both the *de novo* synthesis and in the extrachloroplastic elongation of C_{18} to C_{20} FA did not allow the elucidation of the role of each individual lipid involved in the biosynthesis.

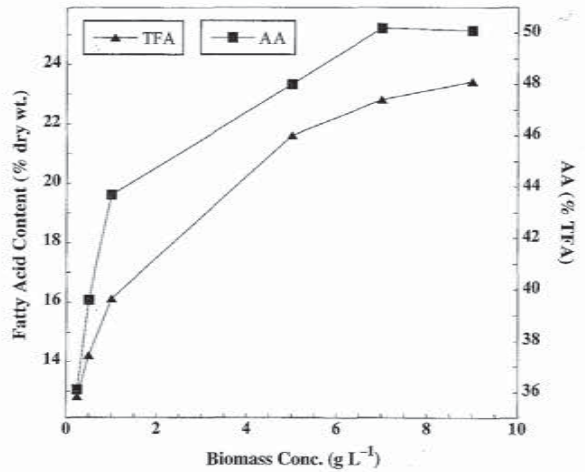
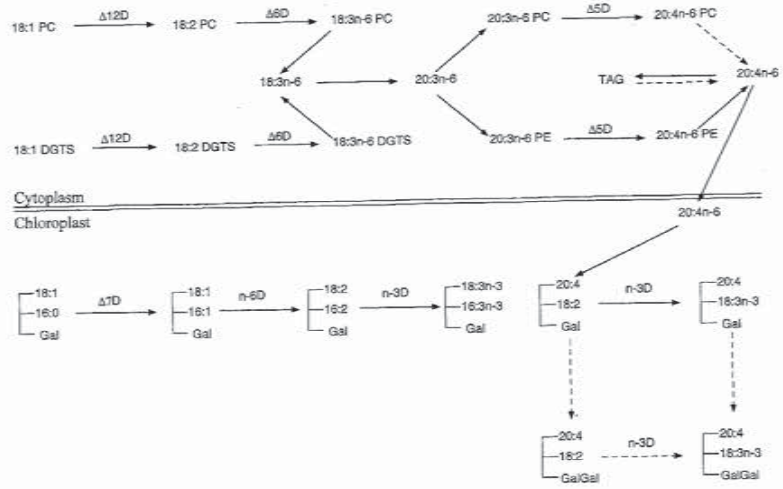


Fig. 4.2. Effect of cell concentration on the fatty acid content and proportion of arachidonic acid (AA) in cultures of *P. incisa* following 3 days of nitrogen starvation. Abbreviation: Total fatty acids, TFA.

Further studies were conducted using [1-^{14}C]oleate. Labeling with [1-^{14}C]oleic acid showed that the first steps of the lipid-linked FA desaturations utilize cytoplasmic lipids (64). PC and DGTS were implicated as the major lipids acting as acyl carriers for the Δ12 and Δ6 desaturations of oleic acid, leading sequentially to linoleic (18:2) and γ-linolenic acid (18:3n-6). As elongation of 18:3 occurs at its carboxylic end, the acyl groups must be detached from their phospholipid carrier in order to be elongated to 20:3n-6. Since labeled 20:3n-6 was detected first in PE and then in PC, we deduced that these lipids (especially PE), are the most likely substrates for the Δ5 desaturation of 20:3n-6 to AA. The presence of molecular species common to both PC and PE, for example AA/AA, 18:2/AA, 18:1/AA, and 16:0/AA, further supports this suggestion (16).

Galactolipids, mostly MGDG, serve as substrates for the chloroplastic Δ12 desaturase; apparently, they are also used for the n-3 desaturation common to higher plants and many green algae. The predominant sequence desaturates the 18:1/16:0 stepwise to the 18:3n-3/16:3n-3 molecular species in a fashion similar to the prokaryotic pathway used by higher plants (50) and some green algae (15). The occurrence of eukaryotic-like molecular species of MGDG and DGDG that contain AA (16) suggests that in *P. incisa* AA is imported from extra-chloroplastic lipids in ways similar to *P. cruentum* (40). An outline of the likely biosynthetic pathways of the PUFA of *P. incisa* is displayed in Scheme 4.2. Interestingly, in many algae (64) desaturation of C_{18} and C_{20} FA involves one or two lipids, usually PC, PE, or diacylglyceryltrimethylhomoserine (DGTS). In *P. incisa*, it appears that all three lipids are involved at different stages in the desaturation processes.



Scheme 4.2. Outline of the biosynthesis of AA in *Parietochloris incisa*.

The inhibitor salicylhydroxamic acid (SHAM) inhibits both the Δ12 and Δ6 desaturations equally (63). In *P. incisa*, SHAM produced a very large increase in 18:1, especially in PC and DGTS, further implicating these lipids as the substrates for the lipid-linked C_{18} desaturations. Another inhibitor, the substituted pyridazinone, SAN 9785, inhibited the TAG assembly in *Pavlova lutheri* (65). In *P. incisa*, Bigogno *et al.* (63) found that this herbicide also inhibited the accumulation of TAG, resulting in a decrease in the share of cell AA that was deposited in TAG from 80 to 52%. Most of the AA that could not be deposited in TAG was exported to the galactolipids, MGDG and DGDG. Their respective share of cell AA drastically increased from 2 and 4 to 10 and 17% (Table 4.8). These findings are in keeping with our hypothesis regarding these lipids as the sink and TAG as the source of AA. Presumably, excess AA can utilize the same vehicle used to mobilize AA from TAG to galactolipids. Due to this inhibition, the proportion of TAG in *P. incisa* decreased from 54 to 25% TFA (Table 4.8). However, the proportion of AA in TAG increased sharply from 40 to 58%. The proportion of AA in PC doubled from 21 to 42% and the percentage of total AA that was deposited in PC increased from 6 to 11%, supporting the suggested role of this lipid as the major donor of AA to TAG. The results of the treatments with SHAM, which inhibited the accumulation of AA but not of TAG, and of SAN 9785, which inhibited the biosynthesis of TAG but not that of AA, indicate that these two processes are not necessarily coupled and are independent of each other.

Role of AA in *P. incisa*

Exposure to high light damages the photosynthetic machinery, particularly at low temperatures. The main site of the damage is the D1 protein of photosystem II. FA desaturation is important for tolerance to intense light, especially at low temperatures, by accelerating the synthesis of the D1 protein (66). The optimal growth temperature of *P. incisa* is 25°C; however, the alga, which was isolated from the slopes

TABLE 4.8
Effect of the Herbicide SAN 9785 on the Distribution of AA in Lipids of *P. incisa*

	SAN 9785	Lipid							
		MGDG	DGDG	SQDG	PG	PC	DGTS	PE	TAG
Lipid (% TFA[a])	-	7	10	8	2	8	5	2	54
	+	18	21	12	7	7	5	3	25
AA (% TFA)[b]	-	9	12	4	4	21	17	37	40
	+	15	23	1	0.3	43	26	34	58
% of all AA[c]	-	2	4	1	0.3	6	3	3	80
	+	10	17	0.5	0.1	11	5	4	52

[a]Share of total cell fatty acids.
[b]AA, % of total fatty acids in particular lipid.
[c]% of total cell AA.
Abbreviations: Phosphatidylglycerol, PG; sulfoquinovosyldiacylglycerol, SQDG.
Source: Reference 63.

of a snow mountain in Japan (53), can withstand temperatures as low as 4°C. Measuring O_2 evolution and variable fluorescence, we found that *P. incisa* is more resistant to low temperatures than *Chlorella*; its resistance is almost as high as that of cryophyte *Chlamydomonas nivalis* (Vonshak, A., Cohen, Z. and Khozin-Goldberg, I., unpublished work).

When stationary cultures of *P. incisa* were labeled with [1-^{14}C]AA and resuspended in fresh medium, the label in lipids decreased primarily at the expense of TAG; this indicates that under normal conditions and at the optimal temperature TAG are predominantly used as a source of energy. When the cultures were shifted from 25 to 4°C, the label in TAG was turned over to polar lipids (63). In another experiment, the molecular species composition and content of MGDG were analyzed after N-starvation and then two days after recovery in full medium (Table 4.9). The share of C_{20}-containing molecular species increased from 9.9 to 47.5% of total MGDG. The content of molecular species of the 20/18 and 20/20 series increased from 1.4 to 11.8 and

TABLE 4.9
Changes in the Distribution and Content of the Molecular Species of MGDG in *P. incisa* Following Recovery from N-Starvation[a]

	Molecular Species Distribution and Content			
	N-Starvation		Recovery	
Molecular Species	% TFA	μg/mL	% TFA	μg/mL
C18/C16				
18:2/16:2	31.3	5.0	2.6	0.8
18:3n-3/16:2	16.0	2.6	4.9	1.5
18:2/16:3	12.5	2.0	4.3	1.3
18:3/16:3	24.2	3.9	25.4	7.7
Total	83.9	13.4	37.1	11.3
C18/C18				
18:1/18:2	tr	tr	3.8	1.2
18:2/18:2	2.0	0.3	6.4	1.9
18:1/18:3n-3	0.8	tr	tr	tr
18:2/18:3n-3	2.1	0.3	2.8	0.8
18: 3n-3/18:3n-3	1.3	0.2	2.4	0.7
Total	6.1	1.0	15.3	4.7
C20/C18				
20:4/18:1	2.4	0.4	10.1	3.1
20:4/18:2	4.4	0.7	21.2	6.5
20:4/18:3n-3	2.1	0.3	7.2	2.2
Total	8.9	1.4	38.5	11.8
C20/C20				
20:4/20:4	1.0	0.2	9.0	2.8

[a]Cultures of *P. incisa* were kept on N-free medium for 14 d. The cultures were resuspended in full medium and cultivated for another 2 d at 24°C. Positional analysis was not performed.
Source: Reference 61.

from 0.2 to 2.8 μg/mL, respectively. Molecular species of the 18/16 series were more desaturated but did not increase in content.

These findings support the hypothesis that one of the roles of AA-rich TAG in *P. incisa* is to serve as a reservoir that can be rapidly used to construct PUFA-rich chloroplastic membranes, especially at low temperatures at which *de novo* PUFA synthesis would be very slow. Indeed, it was recently suggested that the subcellular role of lipid bodies is much more complex than acting as relatively inert carbon stores (67). Recent progress characterizing these organelles allowed the recognition of their important biological roles, such as serving as building blocks for remodeling polar lipids (67).

Conclusion

According to the prevailing theories (18,37), plants accumulate TAG to store energy. Indeed, TAG of most algal species contain mostly saturated and monounsaturated FA; these are less complicated to produce and provide more energy. Our studies of the red microalga, *P. cruentum*, have shown that VLCPUFA accumulated in TAG can be utilized to biosynthesize the eukaryotic-like molecular species of chloroplastic lipids, especially at low temperatures. We have hypothesized that some algae whose habitat is characterized by rapidly changing environmental conditions can swiftly adapt by mobilizing VLCPUFA from their TAG to chloroplastic lipids. Based on this hypothesis, a chlorophyte microalga, *P. incisa* was isolated. This alga is the richest plant source of the nutraceutically valuable PUFA, AA. While the alga can withstand very low temperatures, its optimal growth temperature is 25°C. Maximal accumulation of AA is at the latter temperature, at relatively high biomass concentration and especially under nitrogen starvation. Under these conditions, the proportion of AA can reach 60% TFA and the AA exceeds 20% dry weight. To the best of our knowledge, this is the highest reported content of any PUFA, let alone AA, in algae. Radiolabeling studies have shown that labeled AA was transferred from TAG to polar lipids on sudden cooling to low temperatures; this indicates that TAG of *P. incisa* may indeed have a role as a depot of AA that can be incorporated into the membranes, enabling the organism to quickly respond to low temperature-induced stress.

The finding that most AA of *P. incisa* is deposited in TAG is of practical value, since TAG are the preferred chemical form to introduce AA into baby formula. The capacity for AA accumulation makes *P. incisa* one of the best candidates for large-scale production of AA. *P. incisa* could also be utilized to produce high purity AA for pharmaceutical purposes. Understanding the mechanisms that result in the accumulation of AA in TAG could lead to the identification of other algal species rich in LCPUFA.

Acknowledgments

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Chapter 5

Arachidonic Acid: Fermentative Production by *Mortierella* Fungi

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Arachidonic Acid

Arachidonic acid (AA) is a long-chain polyunsaturated fatty acid (LCPUFA) with twenty carbon atoms and four double bonds (Fig. 5.1). Its systematic name is (all-*cis*)-5,8,11,14-eicosatetraenoic acid (ETA)—in shorthand notation 20:4n-6. It is an important structural component of the lipids in the central neural system, including the brain. AA is also a biosynthetic precursor to several classes of biologically active metabolites, such as eicosanoids.

At present, the commercial demand for AA is dominated by its application in infant formula. Human milk contains significant amounts of two LCPUFA: AA and docosahexaenoic acid (DHA) (22:6n-3). There is a significant body of evidence showing that the neural development in the growing infant may benefit from the provision of these fatty acids (FA), either through breast feeding, or through their inclusion in infant formula. This is most apparent in underprivileged infants and those born prematurely.

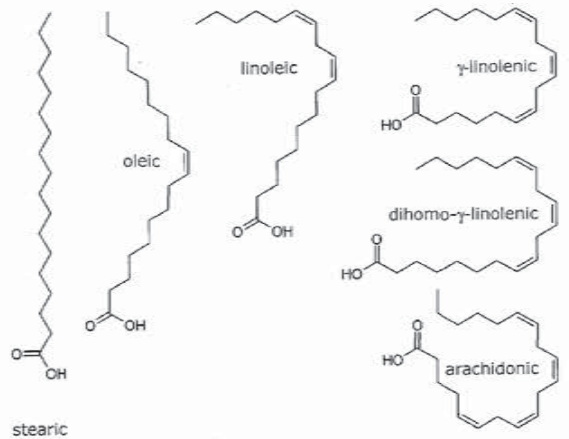


Fig. 5.1. Chemical structures of AA and biosynthetic precursor fatty acids (FA).

The demand for AA for infant formula is growing rapidly, particularly since the Food and Drug Administration in the United States has recently allowed this application. Other applications are also considered, for instance dietary supplements for pregnant women and nursing mothers. LCPUFA supplementation may also benefit a range of neural disorders that are associated with disturbed FA profiles (1,2), but specific applications have not yet been developed. There are also applications in animal feed (aquaculture) and pet food. For fish, AA is an essential nutrient for development after hatching and for larval and fry growth (3). For carnivorous mammals, such as cats, AA is an essential nutrient as well (4). Finally, applications in plant cultivation have also been considered (5).

Sources of AA

Plants do not contain significant levels of LCPUFA. In the case of AA, well-accepted and concentrated animal sources are also not available, even though most of the AA in our diet does come from animal-derived foods. Therefore, microbial sources have been sought and found.

In 1964 (6) it was reported that AA accounted for over 25% of the FA in *Mortierella renispora* lipids. This early report is easily missed because the data are "hidden" in a paper on a different subject. Maybe for this reason, *Mortierella* was not mentioned in an early overview of polyunsaturated FA (PUFA) in fungi (7), although AA production by other fungi, algae, and Oomycetes (microorganisms currently no longer classified as fungi) was reported there. The data from 1964 were included in a massive review of fungal lipids in 1974 (8); this made the information easily accessible. From that moment on, the genus *Mortierella* was an obvious place to search for AA-producing strains because it was there that the highest levels of AA had been found.

When researchers started looking for sources of AA in the 1980s, notably in Japan by Lion Corporation (9) and Suntory (10), and in a number of other groups, they included many species of *Mortierella*, along with other Zygomycetes (such as *Entomophthora*) and Oomycetes (such as *Pythium*). It became apparent that the vast majority of strains currently considered to belong to the genus *Mortierella* produce AA (11), and that *Mortierella alpina* stands out as a high producer (Fig. 5.2, 12), with AA often exceeding 50% total FA (TFA). Moreover, it has been shown to be a safe organism to produce food ingredients (13).

Finally, this organism shows a high ability to accumulate intracellular lipids—mainly triacylglycerols (TAG) (14)—under appropriate conditions (15). The AA is found both in the polar and in the apolar (TAG) lipids. The TAG fraction is used for the commercial product. More recently, algae have also been explored as potential producers of AA (16; also see Chapter 4).

Some Properties of *M. alpina* and *M. alpina* Lipids

Mortierella is a genus of filamentous fungi within the Zygomycetes (17). This group of fungi has a characteristic sexual cycle comprising zygospores and specific

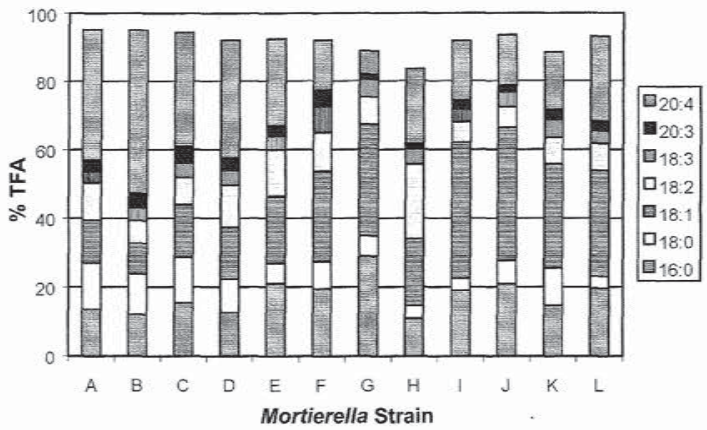


Fig. 5.2. Major FA of various *Mortierella* strains. Total fatty acids, TFA.

pheromones (18), as well as a vegetative (asexual) sporulation cycle. It is also characteristic that the mycelia do not contain septated cells. Rather, the mycelium is a tube that has sections filled with cytoplasm and sections that are empty. The cytoplasm harbors multiple nuclei, even in vegetative spores, and there is no uninucleate stage in the life cycle. This complicates the selection and maintenance of strains, because even a single cell is a population rather than a true individual.

As with many fungi, mycelial growth in liquid culture can be either dispersed or in pellets. Pellet cultures have a (desirable) low viscosity, but may suffer from poor mass transfer into the pellets and degeneration of biomass in their interior (19). The morphology is influenced by environmental conditions, such as the concentration of the carbon source (20). The aseptate nature of the zygomycetous mycelium has led to the fear that these fungi could be particularly sensitive to shear stress in fermentors. Damage to the cell wall could lead to the loss of cytoplasm in an extended section, similar to a ship without watertight bulkheads sinking faster than one that has separate compartments. Indeed, it has been found in a mixed pellet/dispersed culture of *M. alpina*, that most of the AA accumulation occurred in the pellet fraction (21). This has been interpreted as the mycelial fragments having been "shaved" off the pellets by the shear stress, and getting damaged in the process.

The morphological properties of *M. alpina* are quite variable between different isolates. This applies both to the tendency to sporulate on solid media—a desirable property for strain selection and maintenance—and to the tendency to grow dispersed or as pellets in liquid culture. The dispersed morphology is much less common in standard media.

The FA spectrum of *M. alpina* lipids is dominated by intermediates of the biosynthetic pathway leading to AA. The profile seems to be strongly influenced by the

intrinsic properties of the biosynthetic pathway, since different nitrogen sources give surprisingly similar FA percentages in shake flasks (Fig. 5.3), even though the production of biomass and the absolute production level of AA differ by a factor of 10 between the highest- and the lowest-producing conditions.

Nevertheless, the FA composition shows a pronounced kinetic effect; in older, nongrowing cultures, the AA percentage increases compared to younger cultures (Fig. 5.4). This increase is most pronounced in the TAG (storage lipid) fraction (Fig. 5.5, 22,23). It occurs during active sugar consumption, in a phase in which no net lipid-free biomass is being produced due to exhaustion of the nitrogen source. In this phase, the rate of TAG accumulation is very high. It may be that the desaturases and elongases cannot completely match this rate and need some time to produce AA as the end point of the pathway. This will cause a temporary accumulation of its biosynthetic precursors.

Of these precursors, the level of dihomo-γ-linolenic acid (DGLA, 20:3n-6) is remarkably low. This suggests that its synthesis, by the elongase, may be a limiting step in the biosynthetic chain (24), and it certainly suggests that its conversion by the Δ5 desaturase is not. However, very little is known about the actual control structure of the biosynthetic pathway. The usual representation of LCPUFA biosynthesis as a linear chain of free FA is an oversimplification. The FA are not participating as free molecules, but as components of more complex phospholipids. Thus, they participate in a metabolic network that involves various phospholipid and acylglycerol species. It is clear from Figure 5.5 that changes in the FA composition in the various lipid compartments may be quantitatively different. Hence, the transfer of a particular FA from one compartment to another may also be a controlling step (25).

The involvement of different compartments in LCPUFA biosynthesis may also be inferred from the positional distribution of FA in TAG (26). In high-AA oils, its

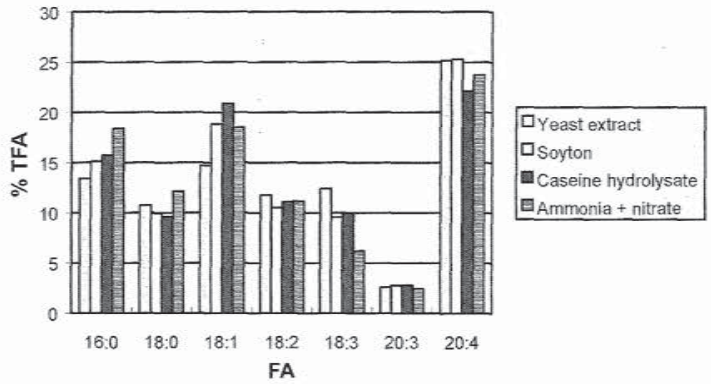


Fig. 5.3. FA profile of *Mortierella alpina* PUF101: various N-sources.

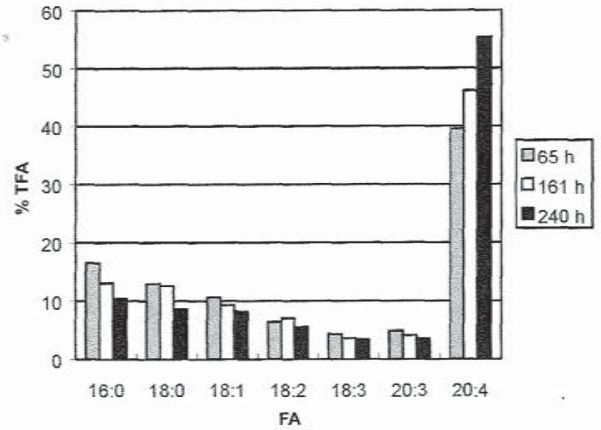


Fig. 5.4. Kinetics of FA profile of *M. alpina* PUF101.

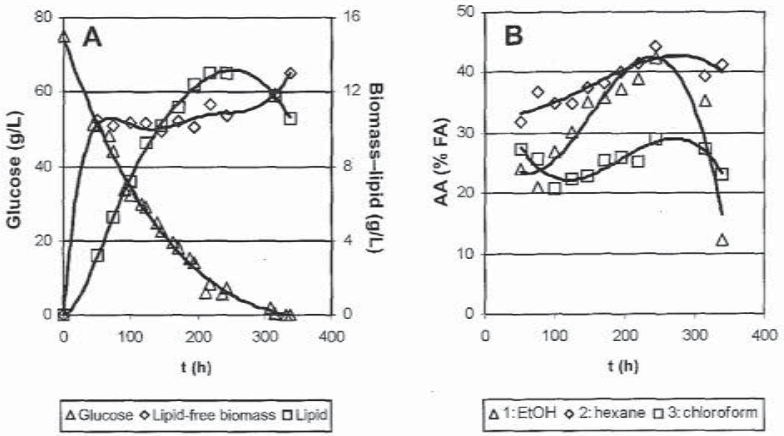


Fig. 5.5. Production kinetics of *M. alpina* PUF101 in shake flasks. (A) Glucose consumption and biomass and lipid production; lipid-free biomass is calculated as the difference between culture dry weight and intracellular lipids. (B) Arachidonic acid (AA) percentage in successive solvent extracts.

precursor FA are found predominantly on the *sn*-2 position of the glycerol moiety. AA itself, however, is found mainly at the *sn*-1 position; this implies that there is more to its biosynthesis than the desaturation of DGLA bound at a certain position, and that

translocation plays a role as well. The picture is further complicated by a different oil sample with a much lower AA percentage, which was analyzed by Myher *et al.* (26), in which the AA was randomly distributed on the glycerol moiety. Nevertheless, this also requires translocation at some stage, because the *sn*-3 position is not available for AA biosynthesis, which is believed to occur on a phospholipid scaffold.

TAG oils with high levels of LCPUFA may have some special properties. An AA content of 40% TFA implies that about 20% of the TAG species must contain two AA residues if all of the remaining 80% contain one. At an AA content of 50%, this fraction has increased to 50% of all TAG. Such species are considered to be poor substrates for most lipases (25). This may explain the extremely high accumulation of AA under some conditions, if multiple AA-substituted species represent metabolic "dead ends."

Older work by the Japanese groups has shown that very high AA levels are found when the fermentation is extended beyond exhaustion of the carbon source. In the 1980s, a resting phase of several days was inserted between the fermentation and oil harvesting, in order to increase the percentage of AA (10). Since it is associated with lipid breakdown this maturation usually does not give a higher AA productivity, but it can be useful when oil with the highest possible AA content is desired. Indeed, extremely high percentages of AA are possible (27).

Shimizu's group at Kyoto University—in collaboration with Suntory—has shown that intermediates of the biosynthetic pathway to AA and a range of other LCPUFA can be produced in this organism (28–30), by changing the culture conditions, using metabolic inhibitors, and/or specific mutations. For instance, the use of a Δ5 desaturase-defective mutant leads to the production of DGLA (27), whereas low temperatures promote the formation of eicosapentaenoic acid (EPA) (20:5n-3) (26). The use of mutants defective in "early" enzymes in the pathway combined with a high activity of "late" enzymes may lead to the accumulation of LCPUFA with unsaturation patterns not commonly encountered in nature (28). Such FA are normally only found as minor components of oils and are formed because the specificity of the biosynthetic enzymes is not absolute.

LCPUFA Biosynthesis in *M. alpina*

In fungi, unsaturated FA are formed from stearic acid (18:0) by integral membrane-bound FA desaturases, which sequentially insert double bonds, and an elongase (31). The desaturases (32) require oxygen and cytochrome b_5 as a cofactor, like other microsomal desaturases from plants and yeast. Electrons are transferred from reduced nicotinamide adenine dinucleotide-dependent cytochrome b_5 reductase to the desaturase *via* the cytochrome b_5.

The desaturases have been difficult to purify in an active form, mainly due to their hydrophobicity. However, some of their properties are known. They contain eight highly conserved histidine residues that are essential catalytically. The consensus sequence is sufficiently robust to use degenerate primers to pick up new desaturase genes.

The first desaturation step is catalyzed by the Δ9-desaturase and uses stearoyl-CoA as a substrate. Subsequent desaturation steps are believed to occur on phospholipid fatty acyl chains. The enzyme acyl-CoA:1-acyl-lysophosphatidylcholine acyltransferase is responsible for channeling FA to the *sn*-2 position of phosphatidylcholine (PC) for desaturation and PUFA production.

During the 1990s classical mutants were isolated; these mutants were defective (33,34) or enhanced (35) in one or more of the desaturase activities. By 2000, the structural genes of the desaturases, the elongase, and some auxiliary genes had been cloned from *M. alpina* thanks to the efforts of various research groups. Their functionality has generally been assessed by expression in a heterologous, non-LCPUFA–producing host (Table 5.1).

This heterologous expression is equivalent to a reconstitution of a section of the AA biosynthetic pathway. Using genes from different sources (including *M. alpina* genes), it has proved possible to detect AA production in *S. cerevisiae* from exogenously supplied linolenic acid (18:2n-6) (31,49).

To achieve a high productivity of LCPUFA from inexpensive carbon sources, such as glucose, it would be advantageous to start with an oleaginous (lipid-accumulating) microorganism as the host for genetic engineering. Unfortunately, genetic systems for such organisms have not yet been as extensively developed as the more conventional yeasts and filamentous fungi. Recently, progress has been made toward genetic transformation of *M. alpina* (50,51). This could become an important tool to influence the flux of carbon through the biosynthetic pathway toward AA and other LCPUFA. Another recently developed tool in the study of the biosynthetic pathway is a cell-free lipid assembly in microsomal membrane preparations obtained from *M. alpina* (52).

TABLE 5.1
Enzymes and Genes from *Mortierella alpina* Involved in Arachidonic Acid Biosynthesis

Enzyme	Substrate	Product	Name	Remarks	References
Δ9 Desaturase	18:0	18:1n-9	ole1p, ole2p, Δ9-3	Expressed in *Saccharomyces cerevisiae* and *Aspergillus oryzae*	36–38
Δ12 Desaturase	18:1n-9	18:2n-6	MaΔ12	Expressed in *S. cerevisiae* and *A. oryzae*	39,40
Δ6 Desaturase	18:2n-6	18:3n-6	Δ6I, Δ6II	Expressed in *A. oryzae*	40–42
Elongase	18:3n-6	20:3n-6	GLELO	Expressed in *S. cerevisiae*	43
Δ5 Desaturase	20:3n-6	20:4n-6	M.A5	Expressed in *S. cerevisiae* and canola	44,45
Cytochrome b_5				Expressed in *Escherichia coli*	46
NADH-Cytochrome b_5 Reductase			Cb5R-I, Cb5R-II	Expressed in *A. oryzae*	47,48

Fermentation of *M. alpina*

A review of the fermentation conditions relevant for AA production with *M. alpina* has recently been published (53); the reader is referred to this for a more in-depth treatment of this subject and for additional literature references.

Generally, microorganisms show the highest accumulation of TAG under nitrogen limitation. The production of AA oil by *M. alpina* is no exception. The dosage of the nitrogen source determines the amount of productive (lipid-free) biomass that can be formed (54). This level should be tuned to the technological limits of the equipment. The carbon dosage then determines how much oil can be accumulated by this biomass. Although *M. alpina* is able to tolerate fairly high glucose concentrations (55), very high levels of carbon dosage may cause inhibitory effects; there is usually an optimal C/N ratio (56). The best way to avoid inhibitory effects is achieved by dosing the carbon as a non-limiting feed.

The concept of the C/N ratio has been persistent in the literature. However, it has rarely been applied beyond the operational optimization referred to previously. The reason for its persistence may well be that lipid production is one of the few biotechnological processes that proceeds quite well in a simple batch culture; in such a system the C/N ratio is an important parameter.

However, in physiological terms, the situation is not as clear. The observation that nitrogen limitation induces oil accumulation is well supported. It follows that a minimal C/N ratio is required to achieve this physiological state. In practice much more carbon will be needed to allow a subsequent high accumulation of lipid, but it remains open to question whether the absolute concentration of the carbon source has an effect as well. This may be different for each microorganism, since it depends on the affinity of uptake systems, catabolic enzymes, and sensors for the carbon substrate or its metabolites. If there is a low-affinity step involved, the magnitude of the carbon excess could play a role in determining the rate of oil accumulation. At least in *M. alpina* oil accumulation proceeds quite well at a modest external concentration of the carbon source.

In a dual feed system, the nitrogen and the carbon sources can be fed independently. The N-feed should be controlling the process, whereas the C-feed is regulated to ensure a continuous but moderate excess of the C-source. Once the desired maximum biomass concentration has been reached, the N-feed can be stopped, and the C-feed can be used to extend the lipid accumulation. This can be maintained until overall productivity decreases, or until the desired percentage of AA in the oil is reached.

Figure 5.6 shows two laboratory-scale fermentations executed according to such a regime at two different temperatures. The quotient of CO_2 produced and O_2 consumed or Respiratory Quotient (RQ) is a convenient on-line indicator of the metabolic state. Growth on glucose leads to an RQ of about 1; lipid production increases the RQ, whereas lipid consumption decreases it. All these conditions are encountered during the process. The biomass production is not indicated in the figure, but it was mostly complete within 80 h.

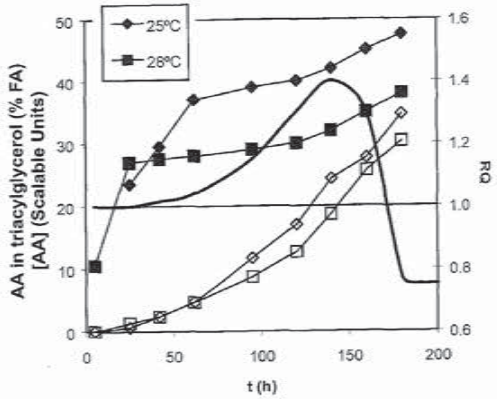


Fig. 5.6. Lab-scale fermentation and the influence of growth temperature. Open symbols indicate AA production (in scale-invariant arbitrary units; left axis). Closed symbols indicate AA percentage of TFA (left axis). The bold line indicates the Respiratory Quotient (RQ, right axis).

It is clear that the lower temperature led to a higher proportion of AA in the lipid fraction. However, the actual AA productivity was not much higher due to the lower metabolic rate, both intrinsic and imposed by limitation of the cooling capacity. The observation that the growth temperature influences the FA spectrum has also been made for other strains of *M. alpina* (28).

As the drop in the RQ shows, the organism is capable of metabolizing the lipid that has been formed. This can be employed as a method to increase the AA-content of the oil—as mentioned previously—but it is also a potential problem, breaking down the lipid after fermentation has been stopped. It is therefore important to inactivate the biomass as quickly as possible after the production phase. Since this may be considered to be the first step of the downstream processing, this subject is dealt with in the chapter on oil extraction (see Chapter 13).

At present, the AA production process with *M. alpina* is the only example of a commercially successful fungal lipid. An increasingly filled molecular biological toolbox would allow efficient production of a whole range of relevant LCPUFA in this organism and other fungi. It is to be hoped that commercial demand will drive the development of such processes to further exploit this fascinating and impressive biochemical pathway.

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Chapter 6

Production of Single Cell Oils by Dinoflagellates

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Introduction

The very long-chain polyunsaturated fatty acid (VLCPUFA) docosahexaenoic acid (DHA, 22:6n-3) is usually associated with marine fish oil. However, marine fish do not synthesize this fatty acid (FA) *de novo*, lacking key enzymatic activities required (1). Instead, fish obtain DHA, along with eicosapentaenoic acid (EPA, 20:5n-3), another VLCPUFA, from their diet. The major primary producers of both of these nutritionally significant VLCPUFA appear to be marine microalgae, of which the dinoflagellates are second only to diatoms as primary producers in coastal waters (2).

Large-scale fermentation technology makes it possible to bypass the marine food chain and produce VLCPUFA-rich oil directly from the microbial primary producer. However, this is an expensive undertaking and results in very high cost oil. Whereas oils derived from plant and animal ("traditional") sources cost ≈ $400–800/ton (3) the cost of the DHA-rich oil obtained from the dinoflagellate *Crypthecodinium cohnii* (DHASCO) is $200/kg.

Producing VLCPUFA-rich oil directly from the microbial source rather than the fish at the top of the food chain has certain advantages that can offset the high cost of the fermentation technology required for large-scale cultivation of axenic cultures of marine microbes. The FA profile of fish oil reflects diet, rather than the capacity, of the fish to synthesize FA. Therefore the profile of fish oil is complex (Table 6.1) and can vary depending on where and when the fish are caught. Due to their complex profiles, fish oils contain a gamut of other FA (both saturated and unsaturated) along with those of nutritional and/or therapeutic significance. As a consequence, the content of the desired VLCPUFA is often relatively low in fish oil. DHA often accounts for less than 10% of the FA in fish oil. Furthermore, fish oils contain several different VLCPUFA; of particular significance is the coincidence in fish oil of the two n-3 VLCPUFA, DHA and EPA. In comparison, microbial oils are simple in their FA profile and can be very rich in the desired PUFA; for example, the dinoflagellate *C. cohnii* produces a cell lipid that can contain >30% w/w DHA (5,8).

Since microbial oils are produced *via* fermentation technology in large contained cultivation tanks under closely controlled and monitored conditions, these oils can be ensured to be of both quality and quantity. Fermentation runs last a matter of days, so production can be increased or decreased to match market requirements. Fish oil sup-

TABLE 6.1
Fatty Acid Profiles of Dinoflagellates and Oils from "Traditional" (i.e., non-microbial) Sources

	Dinoflagellates					Fish Oil	Vegetable Oil	Animal Oil
Fatty Acid	*Gymnodinium splendens* (4)	*Protocentrum minimum* (4)	*Crypthecodinium cohnii* (5)	*Gonyaulax digenesis* (4)	*Cochlodinium heterolobatum* (4)	Cod Liver Oil (6)	Canola Oil (7)	Lard (6)
16:0	29	23	20	22	33	13	3	25
16:1	—	1	1	2	4	6	—	3
18:0	3	1	1	2	1	2	1	13
18:1	10	4	14	8	6	27	64	43
18:2	3	5	—	tr	2	10	22	11
18:3n-6	—	—	—	—	—	—	—	—
18:3n-3	tr	tr	—	2	tr	3	8	1
18:4n-3	2	2	—	1	1	—	—	—
18:5n-3	11	23	—	8	8	—	—	—
20:1	—	—	—	—	—	—	1	—
20:4n-6	—	—	—	—	—	1	—	—
20:5n-3	8	5	—	8	11	10	—	—
22:5n-3	—	—	—	—	—	1	—	—
22:6n-3	30	25	30	25	28	5	—	—

[a]Abbreviation: less than 1% of total fatty acids, tr.

ply is far more difficult to predict, especially in the long term, with the declining fish stocks in many of the world's oceans. It has been predicted that within the next five years, fish oil supplies will be severely limited (1). Fears of contamination from environmental pollutants (such as heavy metals, PCBs, etc.) that apply to fish oils (9–12) do not apply to microbial oils produced in a closed system. Such is the extent of the problems of environmental contamination of marine fish that the FDA has issued several warnings about certain sections of the population (specifically women who are, or could become pregnant) eating marine fish.

Significance of DHA

Of particular relevance to this chapter is the synthesis of DHA by dinoflagellates. DHA is the longest most unsaturated FA found in any significant quantity in nature. Although synthesized predominantly by marine microbes, it is a FA that is important in human development and nutrition (13).

Along with another PUFA, arachidonic acid (AA, 20:4n-6), DHA is a major structural component in human neural tissue, and in particular the gray matter (14). An adequate supply of these two FA has been shown to benefit both brain and eye development in neonates (15–18). Indeed, the consumption of a diet rich in DHA has been linked to the evolution of the highly developed human brain (13). DHA and AA are of such importance that the World Health Organization (WHO), the British Nutritional Foundation (BNF), the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN), and the International Society for the Study of Fatty Acids and Lipids (ISSFAL) have recommended that LCPUFA should be included in all infant formula (19). Crucially, it is also reported that EPA should not be included in infant formula, since this FA is associated with neonate growth retardation (15). Since EPA is contraindicated for infant nutrition, fish oil containing EPA is generally precluded from infant formula in most countries, the exception being certain countries in the Far East.

Neonates deposit a store of DHA *in utero* during the final trimester, obtained from the mother (19), who in turn derives the vast majority of her DHA preformed in her diet (20). Although adult humans have the capacity to synthesize DHA from n-3 polyunaturated fatty acids (PUFA) precursors (α-linolenic acid which is found in plants), this conversion is slow and insufficient to fulfill dietary requirements. After birth, the neonate receives an additional supply of DHA during nursing (19,21). Unfortunately, until the advent relatively recently (20), of formula specifically enriched with DHA and AA, infant formula was devoid of DHA. This was of particular concern for preterm infants who did not receive at least a portion of the DHA supplied in the final trimester. Initially, DHA- and AA-enriched formula was targeted predominantly at preterm neonates; however, the benefit of this supplementation, even for full-term neonates, is so great that presently (just two years after it first entered the market in February 2002) over 50% of all infant formula sold in the U.S. contains a blend of DHA and AA (Fig. 6.1). Formula containing Formulaid™ has been available

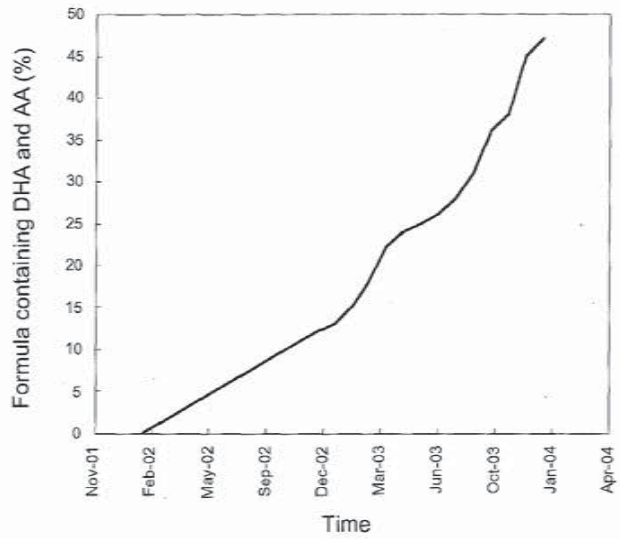


Fig. 6.1. Market penetration of infant formula containing docosahexaenoic acid (DHA) and arachidonic acid (AA) sold in the U.S. The percentage of infant formula sold in the U.S. containing a blend of DHA and AA between November 2001 and April 2004.

in other countries for some time and is currently sold in over 60 countries globally. The acceptance of the new supplemented formula has dramatically increased the sales of DHA, AA, and their blends, which exceeded $100 million in 2003.

C. cohnii as Production Organism

There are over 2000 species of dinoflagellates (22) and, as mentioned previously, they play a major role in primary productivity in coastal waters and are at the base of the marine food chain. Morphologically, the dinoflagellates are characterized by the possession of two flagella, one in a groove around the middle of the cell and one that occupies a groove along the axis of the cell. The nuclear organization of the dinoflagellates is unique among eukaryotes in that they lack histones (23) and the chromosomes remain condensed throughout the cell cycle. Another unique feature of the dinoflagellates is their genome size (23), which can exceed the size of the human genome by 100-fold!

As stated previously, it has long been recognized that certain dinoflagellates produce VLCPUFA to varying degrees; one of these FA is DHA (Table 6.1). Many also synthesize EPA, making them less suitable for the production of DHA-rich oil for infant formula supplementation. Furthermore, the majority of dinoflagellate species

are photosynthetic. Although photosynthetic growth may appear attractive because the carbon/energy source (sunlight) is free, this advantage is illusory. The self-shading in photosynthetic cultures precludes high cell densities (24) and the O_2 produced during photosynthesis must be removed. As a result of the low cell densities, enormous closed systems would be required (an open system is unsuitable to produce high-grade food oils destined for neonate nutritional applications) and vast culture volumes would need to be harvested (24). As a result of these limitations, auxotrophic production is not as economical as heterotrophic cultivation in stirred tank fermentors.

However, among the heterotrophic dinoflagellates, *C. cohnii* was identified as a prolific producer of DHA (Table 6.1). This organism was extraordinary in that it produced no other PUFA in its cell lipid in any significant amount, other than DHA. The only other PUFA reproducibly detected in oil from *C. cohnii* is the VLCPUFA 28:8n-3 constituting only ≈1% of total FA (25). Despite the lack of intermediates, the organism was capable of producing over 30% of its cell lipid as DHA (5,8). This DHA-rich oil is accumulated predominately as triacylglycerol (TAG), the preferred form for food use.

Strain Selection and Optimization

Although the production of DHA as a major constituent of the TAG accumulated by *C. cohnii* was reported in the late 1960s and early 1970s (5,26,27), the strains used for these studies were not capable of suitable growth in stirred tank fermentors. These problems were associated with sensitivity of the organism to both the shear and high dissolved O_2 in stirred tank fermentors. Despite the apparent problems in cultivating *C. cohnii*, Martek opted to develop this microalga as their production organism. Using a combination of strain screening and culture condition modification, a process was developed to allow growth and DHA production by *C. cohnii* at the large scale required for commercial application.

Many strains of *C. cohnii* were obtained from public culture collections and screened for growth and DHA production in fermentors. From this screen, a single strain from the University of Texas culture collection was identified as the most promising, based on growth rate and DHA production. This strain became the parent from which all Martek production strains were developed, *via* classical strain selection, although the strains in use today bear little or no resemblance to this original strain. Strain selection and cultivation from single colonies on agar plates as well as yielding potentially improved strains ensures genetic homogeneity of cultures used for production and guards against phenotypic "drift."

Using classical strain screening, many strains have been isolated that have potentially attractive attributes. Screening for increased lipid production, decreased by-product formation, and improved growth under conditions suitable for large-scale production have all been successfully accomplished, although by no means every strain identified in a lab-based screen makes it as far as production! As each new potential strain is obtained, a dedicated group is involved in optimizing the culture media and

conditions to assess and realize the full potential of each strain before the fermentation group takes the strain forward and carries out scale-up trials.

Strains are now available that are not only capable of good growth in stirred tank fermentors but can also grow under the decreased Cl^- concentration required to avoid excessive corrosion of steel fermentation tanks. At the same time as producing strains amenable to large-scale cultivation, the strain selection/optimization program has been very efficient at increasing the productivity of the production strain. One indication of this success is shown in Figure 6.2. Over the 6-yr period from 1997 to 2002, the cell lipid of the production strain (as lipid % w/w dry weight) was increased by 242%.

Industrial Production of DHASCO™

The basic fermentation process used to cultivate *C. cohnii* for the subsequent extraction of cell lipid resembles other commercial fermentation processes in many respects (Fig. 6.3). A cryovial of a certified proprietary strain (developed as described previously) is thawed and used to inoculate a seed train, including shake flask and fermentors, so as to maintain an inoculum volume of 5–10% (v/v) for each successive step up to a final vessel volume of ≈200 m^3. Currently Martek has over 1000 m^3 of fermentation capacity dedicated to DHASCO production in the U.S. Expansion to meet the current demand for DHASCO means the fermentation capacity should exceed 3000 m^3 by the end of 2004. As *C. cohnii* is a relatively slow growing organism, compared to commonly fermented bacteria, the seed train takes a considerable period and so must be carefully

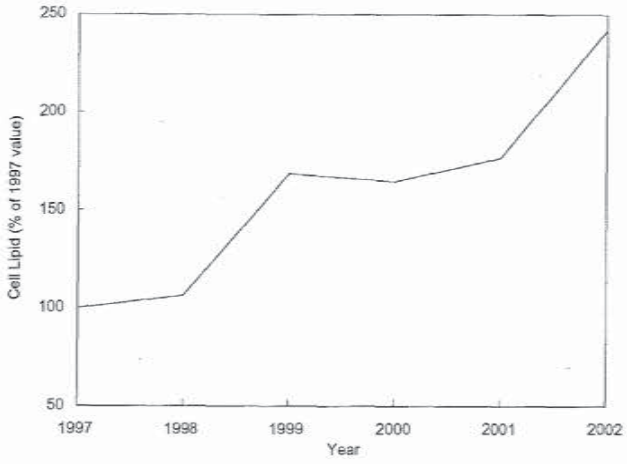


Fig. 6.2. Increase in the cell lipid of *Crypthecodinium cohnii* obtained *via* classical strain selection.

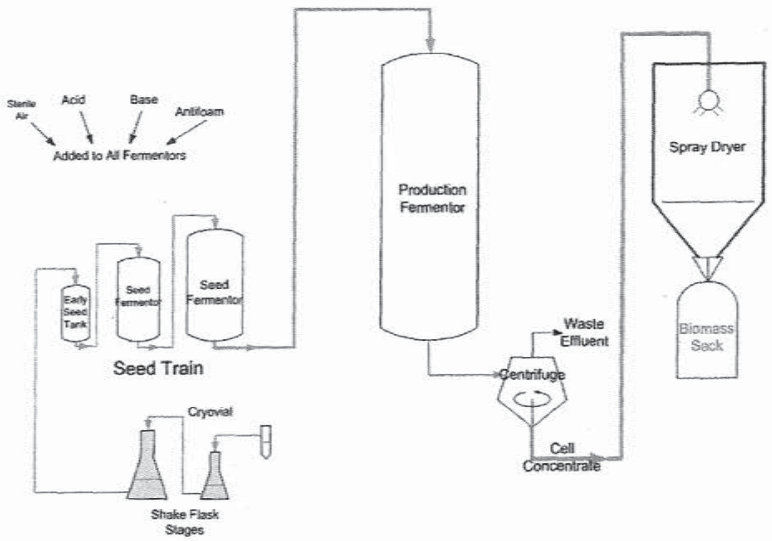


Fig. 6.3. Outline of the fermentation process used to produce the "dinoflagellate" single cell oil (DHASCO™) from *C. cohni*.

coordinated to ensure that inocula are available for each main tank as it becomes available. The slow growth rate of *C. cohnii* in comparison to bacteria also has implications in terms of susceptibility to contamination. As a result, plant hygiene is paramount and "clean-in-place" and "sterilize-in-place" regimes have to be closely monitored and maintained in order to avoid contamination and loss of fermentation batches.

The culture medium throughout the seed train is kept constant so as to avoid stressing the organism and slowing growth further. One significant feature of the medium used at commercial scale is the Cl^- level employed. As a marine microbe *C. cohnii* requires a saline environment in which to grow. The Cl^- of seawater, and seawater-based media, commonly employed in academic research (26,28) is ≈19000 ppm and is not compatible with stainless steel cultivation tanks. As a result, media, and adapted strains, have been developed so that cultivation at only a fraction of the Cl^- concentration of seawater is possible. To further minimize the risks associated with the Cl^- requirement of *C. cohnii* (even at the lower concentrations possible with the current production strain) many of the tanks used by Martek for the cultivation of *C. cohnii* are constructed of higher grades of stainless steel (317L, 2205, or AL6XN).

Significant lipid (TAG) accumulation does not occur during active growth in a nutrient-replete medium; it occurs during idiophase, after a culture nutrient other than the carbon (C) source is depleted. The limiting nutrient is usually nitrogen (N). Therefore, *C. cohnii* fermentation is a C-fed batch and progresses in two stages. The

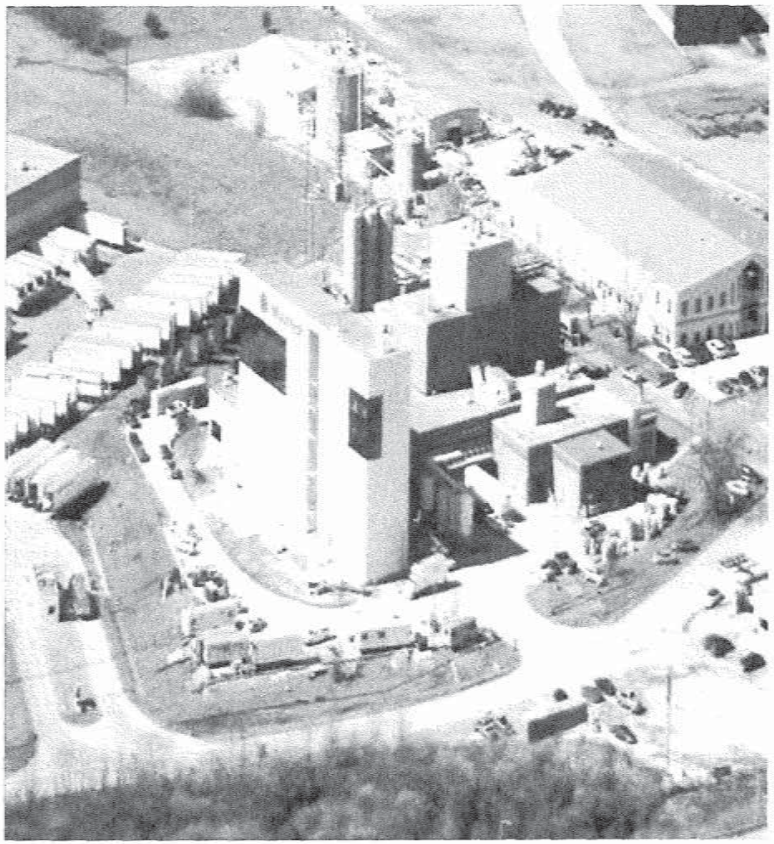


Fig. 6.4. Martek's original production facility in Winchester (KY) used to cultivate *C. cohnii* for DHASCO™.

first is the active growth phase during which the lipid content of the biomass is modest (approximately 20% w/w dry wt), and the cells are motile. Once the N-source is depleted, C is continuously supplied to the fermentor. Since cell growth and division is halted due to the lack of N for *de novo* protein and nucleotide synthesis, the supplied C is converted into a storage lipid (TAG) rich in DHA. During this lipid-accumulating phase *C. cohnii* cells lose their flagella and become "cyst-like" cells packed with DHA-rich lipid bodies (Fig. 6.5). Cell lipids at this stage constitute over one-half the cell dry weight. Maintaining the C-concentration in the cultivation vessel is important to optimize lipid accumulation not only to promote synthesis but also to avoid utilization of the internal storage lipids. Induction of β-oxidation by cells experiencing

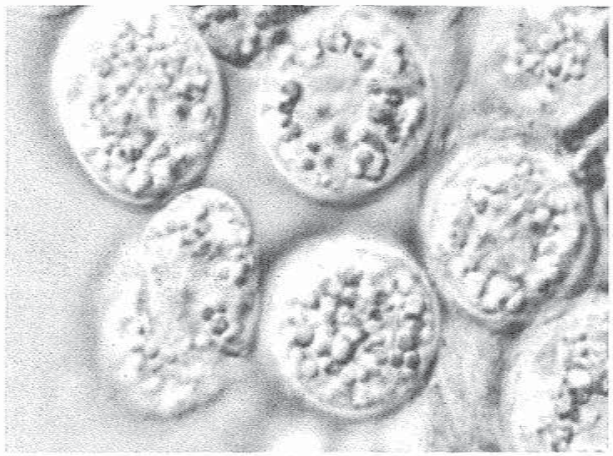


Fig. 6.5. Cells of *C. cohnii* packed with DHA-rich oil bodies.

C-limitation causes an increase in the levels of free FA and di/monoacylglycerols in the final hexane extract; this complicates processing and decreases both final yield and oil stability. The possible induction of β-oxidation at the end of fermentation, during harvest/drying and during storage, is another potential source of oil quality and quantity loss. To decrease the impact of β-oxidation, harvest time must be kept to a minimum and operating conditions must be closely controlled.

Although the strain improvement program has identified a number of improved strains of *C. cohnii*, production of DHASCO at commercial scale using these strains is not always straightforward since *C. cohnii* can suffer from many of the scale-up challenges associated with other production organisms and processes. The very large fermentors employed by Martek have the advantage of being more cost effective than smaller tanks but there are some trade-offs and operational complications. The most obvious change as the culture vessel size increases is the higher absolute pressure at the bottom of the tank. Depending on mixing time and mass-transfer coefficients, this can result in increased levels of dissolved gases that can impact the growth and productivity of *C. cohnii*. Therefore, sufficient mixing is required to avoid the depletion of O_2 and buildup of CO_2 without exposing the organism to excessive shear.

Once the culture fermentation vessel has produced sufficient biomass and lipid it is harvested by continuous centrifugation, which includes a washing step to remove culture components; then the biomass is spray-dried to <10% moisture (Fig. 6.3). Removal of the water stabilizes the lipid-rich biomass and prevents lipid degradation due to biological processes. The temperature during spray drying is another factor that must be optimized to minimize drying time while maximizing oil quality. In general, the lowest temperatures possible are used throughout the processing of the DHA-rich oil in order to avoid compromising oil quality. Oxidation of the intracellular oil by

exposure to O_2 is kept to a minimum by storing the freeze-dried biomass at –20°C and under N_2, until extraction.

Efficient extraction of the biomass is achieved by mixing the dried biomass with hexane to form a slurry that is then passed through a disruptor. This disruption breaks open the *C. cohnii* cells and makes the intracellular lipid bodies more available for extraction. After contact with the solvent, the hexane/oil mixture (miscella) is separated from the oil-depleted biomass and passed on to an evaporator to remove the hexane and yield the crude oil that is stored at low temperature and under N_2 (to avoid oxidation of the PUFA-rich oil) prior to processing (Fig. 6.6).

The crude oil is processed essentially in the same manner that vegetable oil is (Fig. 6.7). Refining, bleaching, winterization, and deodorization yield an orange, translucent oil that, after the addition of tocopherols as antioxidants, is blended with high-oleic sunflower oil (HOSO) to a standardized 40% w/w DHA.

Characteristics of DHASCO™

DHASCO™ is a free flowing oil that contains a standardized 40 wt% DHA (Table 6.2). The processed *C. cohnii* oil contains a higher percentage of DHA, but it is blended to 40% w/w DHA with HOSO to the specified potency. The oil is >95% w/w TAG with the remainder being primarily nonsaponifiable material. In this regard DHASCO is typical of food-grade vegetable oils.

The oil has a low aromatic impact (as defined by Sensory Spectrum, NY), the most prevalent characteristics being a "green/beany" note. Although not as bland as canola or sunflower oil, the flavor qualities of DHASCO lack the strong, undesired "fish" or "paint" notes associated with even the most refined fish oils.

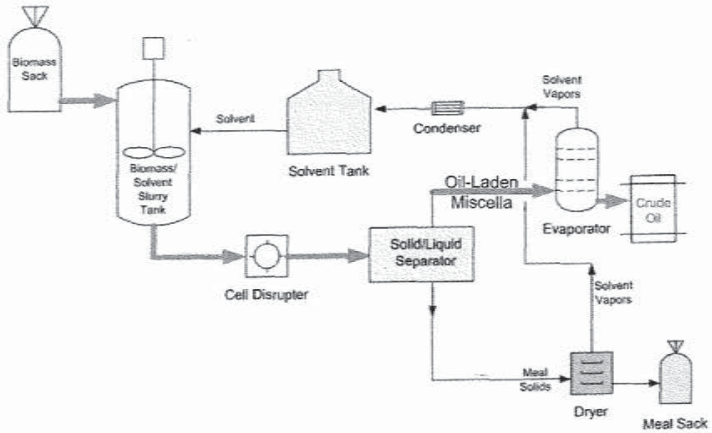


Fig. 6.6. Extraction of triacylglycerol oil from *C. cohnii* to give crude DHASCO™.

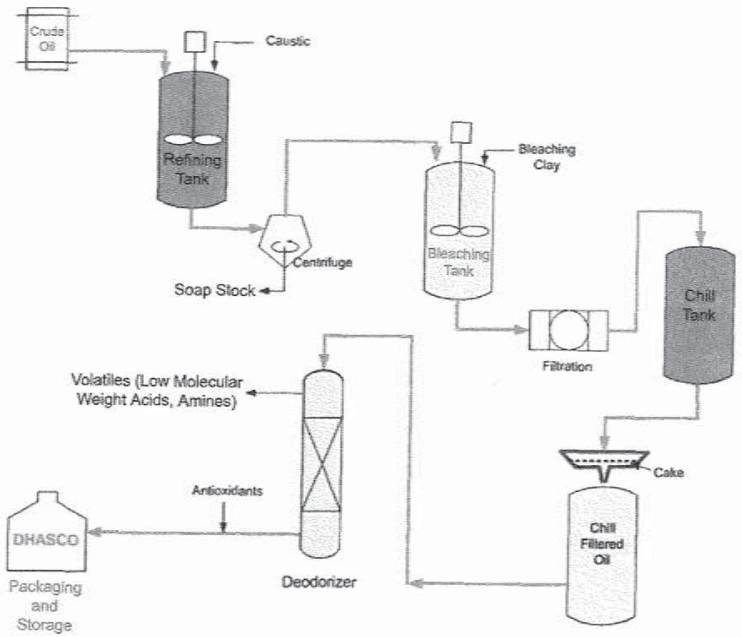


Fig. 6.7. Outline of the refining and processing steps used to produce food-grade DHASCO™.

TABLE 6.2
Fatty Acid Profile of Dinoflagellate Single Cell Oil, DHASCO™

Fatty Acid	Specified w/w%
10:0	0–2
12:0	0–6
14:0	5–20
16:0	5–20
16:1	0–3
18:0	0–2
18:1	10–40
18:2n-6[a]	0–5
22:6n-3 (DHA[b])	40–45
24:1[a]	0–2
Others	0–3

[a]Fatty acids not synthesized by *Crypthecodinium cohnii* but present in DHASCO™ because of their presence in high-oleic sunflower oil.
[b]Abbreviation: Docosahexaenoic acid, DHA.

Analysis has confirmed that DHASCO™ is devoid of heavy metals (arsenic, mercury, and lead). Likewise, pesticide residues are not found in DHASCO™; 74 pesticides were tested for and were not present at detectable levels.

Oil quality is carefully guarded during production and processing; it is maintained in the final product by adding 250 ppm each of ascorbyl palmitate and tocopherols (as antioxidants) and by storing the oil at low temperature in N_2-purged containers. The stability of the oil is such that it has a shelf life of two years from the date of shipping if stored frozen. Regardless of the storage method (either frozen or at room temperature), DHASCO™ will maintain a peroxide value (PV) of <5 (and routinely <1) meq/kg oil for two years if maintained in N_2-purged vacuum bags.

Safety of DHASCO™

Due partly to its novel (microbial) origin and partly due to its intended application as part of a supplement to be added to infant formula, the safety of DHASCO™ was thoroughly examined. It is true to say that DHASCO™ is probably one of the most carefully tested oils, in terms of safety and efficacy (see Chapter 11).

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Chapter 7

Production of Docosahexaenoic Acid by the Marine Microalga, *Ulkenia* sp.

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Introduction

Growing interest in the nutritional and pharmaceutical relevance of long-chain polyunsaturated fatty acids (LCPUFA) has created an increasing demand for these lipids. In particular, long-chain n-3 PUFA are receiving attention because a large number of epidemiological studies, clinical trials, and experimental animal studies indicate that they exhibit health benefits against numerous diseases such as coronary heart disease, inflammatory disease, some cancers, and various mental disorders. In addition, docosahexaenoic acid (DHA, 22:6 n-3), presumably the most important n-3 PUFA, is essential for the development of the brain and the retina of the eye (see Chapter 12).

The major source of long-chain n-3 PUFA are oils of marine fatty fish such as salmon, sardine, mackerel, and tuna. Currently, refined fish oils containing n-3 PUFA are widely distributed. However, they exhibit several drawbacks including a low concentration of n-3 PUFA, potential contamination with environmental pollutants, such as dioxins and PCBs, and fluctuations in quality due to seasonal and climatic variations and changing geographical locations of the catching sites. Other limitations of fish-derived n-3 PUFA oils are their undesirable fishy taste and potential oxidative instability. In addition, it is expected that the supply of n-3-PUFA-rich oils from fish will be inadequate to meet future demand. Thus, numerous activities to exploit alternative sources for the production of long-chain n-3 PUFA have been initiated over the past few years.

Microalgae as a Source of n-3 PUFA

Microalgae have become a promising alternative source of long-chain n-3 PUFA, since specific microalgae species have been identified as the primary producers of these fatty acids (FA). They contain an efficient enzymatic system that synthesizes the FA. Even fish do not synthesize long-chain n-3 PUFA in significant quantities, but rather they incorporate them *via* the food chain from microalgae.

Microalgae exhibit many advantages regarding the production of long-chain n-3 PUFA. Some of them are able to accumulate large amounts of oil with very simple FA profiles with n-3 PUFA being the main component. If heterotrophic strains are

used for production, seasonal and climatic dependences can be avoided. Since the production process occurs under fully controlled constant conditions, consistent quality can be guaranteed and the production capacity can be easily adapted to meet demand. Furthermore, n-3 PUFA oils derived from microalgae are free from any contaminants and exhibit organoleptic properties superior to those derived from fish.

Ulkenia as a Source of DHA

Although numerous species may have the ability to synthesize long-chain n-3 PUFA, few of them have been identified as being suitable producers from an economic point of view. In particular, if one considers heterotrophic production strains that can be grown in conventional stirred fermentors rather than phototrophic strains that require either open pond systems or sophisticated photobioreactor technology only a few candidates remain. One of these is the dinoflagellate, *Crypthecodinium cohnii;* another is *Schizochytrium* sp., which belongs to the order Thraustochytriales. Both organisms have been described extensively (1–6) as potential DHA producers and are being used today for commercial DHA production [(7), see also Chapters 3, 6, and 8].

When the authors started to screen for DHA producers among the heterotrophic microalgae, several selection criteria were defined that had to be fulfilled by potential candidates: excellent growth rates and high productivity; suitable lipid profile; genetic stability; ease in handling (in-strain preservation and maintenance); acceptance of inexpensive, axenic production media; and ability to tolerate shear stress. With *Ulkenia*—so far a completely ignored DHA producer—an organism fulfilling all of the previously mentioned criteria was identified.

Together with several other genera, including *Japonochytrium*, *Aplanochytrium*, *Schizochytrium*, and *Thraustochytrium*, *Ulkenia* belongs to the order Thraustochytriales (8); it is part of the Labyrinthulomycota, a phylum of eukaryotic, heterotrophic protists. *Ulkenia* was described as a new genus by Gaertner in 1977 (9) and is clearly distinguished from all other genera of the Thraustochytriceae. The life cycle consists of several phases and includes a material multicellular stage in the form of an amoeboid protoplast that migrates before differentiating into zoospores following cell divisions.

Mass Cultivation of *Ulkenia*

Ulkenia shows excellent growth on a simple glucose yeast extract medium (Fig. 7.1) and accumulates large amounts of oil intracellularly with DHA, docosapentaenoic acid (DPA, 22:5n-6), and palmitic acid (16:0) being the dominant FA. A number of conventionally used carbon sources are tolerated in high concentrations (>60 g glucose/L). The cells are grown at temperatures around 30°C and are harvested once they stop multiplying and accumulating triacylglycerols (TAG). Subsequently, they are submitted to a number of procedures, including extraction of the crude oil and further refining steps, in order to achieve an edible oil (Fig. 7.2). Currently, the fermentation

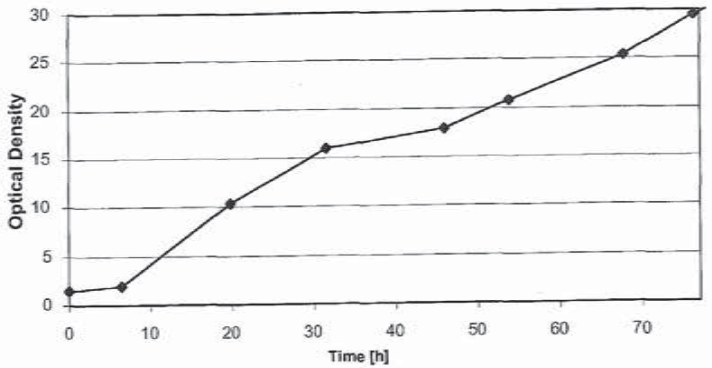


Fig. 7.1. Growth behavior of *Ulkenia* sp. in a laboratory-scale fermentor.

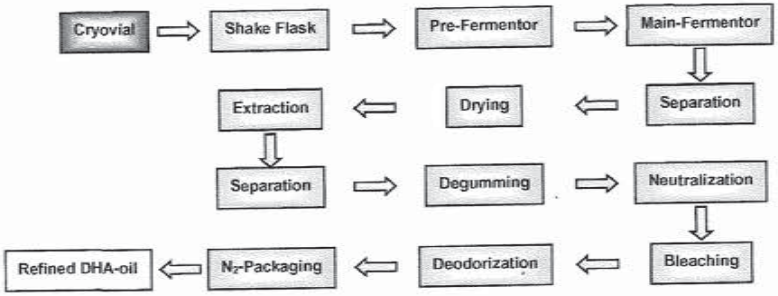


Fig. 7.2. The production process of *Ulkenia*-derived docosahexaenoic acid (DHA) oil. The main fermentor is inoculated *via* a series of pre-cultures. After completion of growth and oil production in the main fermentor, the cells are harvested and dried before the crude oil is extracted from the biomass. The subsequent refining process includes steps of degumming, neutralization, bleaching, and deodorization.

process is carried out routinely on an industrial scale of 80 m^3 using conventional stainless steel reactors.

Properties of the DHA Oil Derived from *Ulkenia*

One of the most remarkable features of the oil is seen in its purity and simplicity when compared to a conventional fish oil. Basically, only three FA represent more than 85% of all FA (Table 7.1). The oil consists mainly of neutral lipids; typically, over 98% are triacylgycerols, and only minor amounts of common phytosterols can be found in the unsaponifable fraction (Fig. 7.3). The most abundant TAG are the DHA-

TABLE 7.1
Typical Fatty Acid Composition of Docosahexaenoic Acid Oil Derived from *Ulkenia*

Fatty Acid	Content (% w/w of Total Fatty Acids)
Myristic Acid (14:0)	2.7
Pentadecanoic Acid (15:0)	1.2
Palmitic Acid (16:0)	29.7
Stearic Acid (18:0)	1.1
Eicosatetraenoic Acid (20:4n-7)	1.0
Docosapentaenoic Acid (22:5n-6)	10.5
Docosapentaenoic Acid (22:5n-3)	1.6
Docosahexaenoic Acid (22:6n-3)	46.0

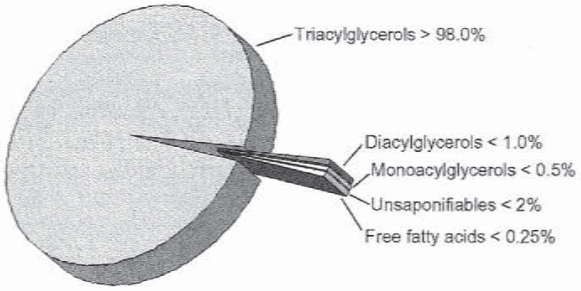


Fig. 7.3. Composition of the docosahexaenoic acid oil derived from *Ulkenia*.

containing TAG (Fig. 7.4). These three species typically account for over 70% of the total TAG.

Depending on the production conditions, the consistency of the oil may vary between being slightly waxy to a clear yellow liquid. The oil is free of contaminants (pesticides, dioxins, and heavy metals) that might otherwise be found in fish oils and other marine products. If stored in an unopened vessel under an inert atmosphere, the oil is stable for over 12 months at ambient temperature and for over 24 months at temperatures <5°C.

Genetics of *Ulkenia*

The productivity of *Ulkenia* sp. may be improved further by classical strain mutation (improvement) and/or genetic engineering techniques. Although at present only wild type strains of *Ulkenia* as isolated from nature are used to produce DHA oil, it is of interest to explore the genetic basis of the impressively high DHA productivity of *Ulkenia*.

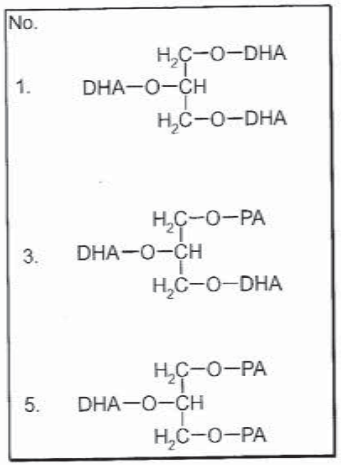


Fig. 7.4. Main triacylglycerol species in the docosahexaenoic acid oil derived from *Ulkenia*.

Basically, there are two completely different biosynthetic pathways known to produce long-chain n-3 PUFA. The more common linear pathway is a stepwise addition of C-2 units by elongases and the introduction of double bonds by desaturases into the FA chain; this starts with palmitic acid, the end product of the FA synthase complex.

However, some microorganisms show a completely different, nonlinear pathway in which long-chain PUFA are produced from activated C-2 and C-3 units by a polyketide synthase (PKS) homolog multienzyme complex. PKS are generally involved in the production of various secondary metabolites, such as antibiotics and aflatoxins, from ketide units (10). A PUFA-producing PKS was first described for *Shewanella*, a marine bacterium rich in EPA (11). Since then, various other organisms, including prokaryotes and eukaryotes, have been identified as producing DHA, EPA, and n-6 DPA *via* a PKS pathway (12). The PUFA-PKS multienzyme complex consists of keto-synthase, malonyl-CoA:ACP acyltransferase, acyl-carrier-protein (ACP), keto-reductase, chain-length factor, acyltransferase, enoyl-reductase, and dehydrase/isomerase.

In *Ulkenia* the authors have also identified the PKS motif. The *Ulkenia*-PKS genetic sequence consists of three open-reading frames (ORF, Fig. 7.5), but the common linear structure of the PUFA-PKS known from other organisms turns out to be disrupted in this organism. The distance between the ORF is unusually large. ORF 3 could not be located in the immediate neighborhood of the other two ORF; this suggests a substantial gene rearrangement. Another significant difference is the large number of ACP domains. Known prokaryotic PUFA-PKS clusters contain only 5 or 6 ACP domains, like the EPA-producing bacterium, *Shewanella* (6 × ACP), and *Photobacterium* (5 × ACP), or the DHA-producing *Moritella* (5 × ACP). The eukary-

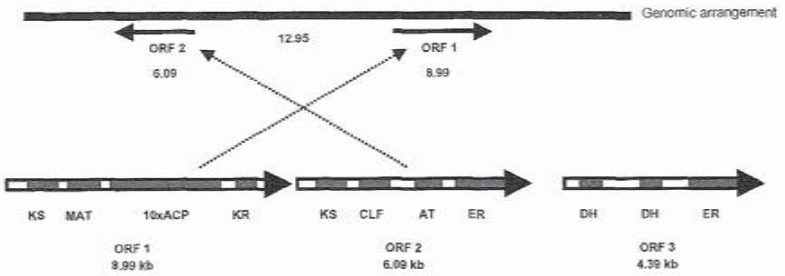


Fig. 7.5. Genomic arrangement of *Ulkenia* polyketide synthase (PKS) gene cluster and open reading frames, ORF; keto-synthase, KS; malonyl-CoA:ACP acyltransferase, MAT; acyl-carrier-protein, ACP; keto-reductase, KR; chain-length factor, CLF; acyltransferase, AT; enoyl-reductase, ER; and dehydrase/isomerase, DH.

ote, *Schizochytrium*, contains 9 × ACP domains, whereas the authors have identified 10 ACP domains in *Ulkenia* (Fig. 7.6). Homologous genes could also be detected in other PUFA-producing *Ulkenia* species by polymerase chain reaction (PCR). The existence of these conserved genes in some prokaryotes, and in distinct eukaryotes, can be interpreted as evidence for a horizontal gene transfer between these organisms.

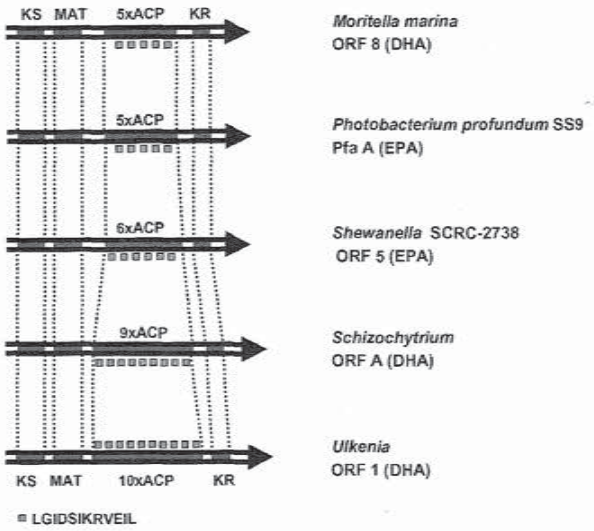


Fig. 7.6. Comparison of *Ulkenia*-PUFA-PKS ORF1 with different homologous ORF of other PUFA-PKS from other microorganisms.

TABLE 7.2
Specification of Docosahexaenoic Acid (DHA) Oil Derived from *Ulkenia*

Specification	Value
DHA (mg/g oil)	>380
DHA (% TFA)	>43
Peroxide value (meq/kg)	<5
Acid value (mg KOH/g)	<0.5
Unsaponifiables (%)	<2
trans Fatty acids (%)	<1

Application of *Ulkenia*-Derived DHA

As is the case with other edible oils, *Ulkenia* DHA is particularly easy to incorporate into products with a high fat content. For example, it can easily be added with other fatty components to enrich margarine or cream cheese. But it also works very well in a variety of other applications, such as bread and cookies, health bars, yogurts, and beverages. For liquid products, such as fruit-based beverages, milk, or soy beverages, the best way to introduce it is to homogenize it directly in the product or to prepare a concentrated emulsion. The high DHA concentration and superior sensory properties provide significant formulation advantages.

Application studies have been conducted in baked goods, cereals, dairy products, spreads, yogurts, fruit juices, and snacks; the sensory evaluation, as well as stability data of DHA-enriched food prototypes, shows very good results. For example, DHA was stable during the standard baking process in breads and cookies. The DHA content in enriched health bars was stable for at least 18 months under regular storage conditions and the taste remained excellent.

Currently, the best known n-3 products are dietary supplements. The high concentration of *Ulkenia*-derived DHA oil makes it possible to produce much smaller capsules than fish oil capsules that still contain the same amount of DHA as the common fish oil supplements and without the fishy aftertaste.

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58

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Chapter 8

Alternative Carbon Sources for Heterotrophic Production of Docosahexaenoic Acid by the Marine Alga *Crypthecodinium cohnii*

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Introduction

In recent years, interest in polyunsaturated fatty acids (PUFA), especially the n-3 PUFA, has increased considerably due to their various physiological functions in the human body and their beneficial effects on human health (1–4). Until recently, fish oils have been the only major sources of these fatty acids (FA); now we have the advent of single cell oils (SCO) with the identification of several marine microorganisms that contain substantial quantities of PUFA. These are now considered to be the major sources of these important FA that are covered in the various chapters in this book.

Of key importance is the heterotrophic marine dinoflagellate, *Crypthecodinium cohnii*, that has been studied intensively (5–12) and represents the only commercial source of docosahexaenoic acid (DHA, 22:6n-3) in an oil in which it is the sole PUFA present (13,14). *C. cohnii* can accumulate a high percentage of DHA [25–60% of the total fatty acids (TFA)] in its triacylglycerols (TAG) with only trivial amounts of other PUFA (see Chapter 6). Important parameters for optimal DHA productivity include growth rate, final biomass concentration, total lipid content, and the DHA proportion of the lipid (13,14). In most of the documented commercial cultivation processes, glucose is used as the carbon and energy source. Glucose, of course, represents an easily accessible feedstock for many industrial fermentation processes and is usually obtained, in the form of glucose syrups, from the hydrolysis (chemical as well as enzymological) of corn starch. It is inexpensive, readily available, can be stored as a concentrated solution, and is water-soluble so that fermentation media can made and sterilized without difficulty and then transferred into the final fermentor as a single solution. However, it is not the only possible substrate that could meet these criteria. This chapter discusses the use of alternative carbon sources, acetic acid and ethanol, on growth, lipid accumulation, and DHA productivity of *C. cohnii* in fed-batch cultures.

Acetic acid is produced by the petrochemical industry on a very large scale. It costs about three times that of glucose at about $450/t. Its drawback for large-scale

fermentation use is that in concentrated form it requires careful handling; any spillage or contact with the skin must be dealt with promptly. It is not, however, classed as a "strong" acid and is not as aggressive as an inorganic acid, such as HCl or HNO_3. Therefore, it does not need the stringent precautions that would be needed in handling large quantities of these acids. In a fermentor its concentration will be very diluted, and hazards at this level of the operation will therefore be minimal.

Ethanol can be produced by fermentation; this is performed extensively in countries such as Brazil and New Zealand, though extensive "gasohol" programs to produce fuel-grade ethanol by fermentation also occur in the U.S. However, since it is produced by glucose fermentation (originating from corn starch or from the sucrose in sugar cane), its cost will be higher than that of glucose. Ethanol is also produced by the petrochemical industry and, as fuel-grade material, sells for about $160/t. "Food-grade" ethanol, which would be needed for producing "food-grade" DHA, sells at $370–400/t and is about 80–90% of the price of acetic acid. Its main disadvantage is its flammability when stored and transported around a production site in its undiluted form. Also, and not of insignificant consequence, processes using ethanol may need to be continuously scrutinized by regulatory authorities to prevent the use of the ethanol for purposes other than for which it was intended. This may then place unwanted restrictions on its suitability as a fermentation feedstock.

Both substrates, like glucose, are water-soluble and therefore present no problems in mixing in the fermentation medium. Both substrates are also "pure" and are appropriate for production of products destined for human consumption. They have no residual nonfermentable components so the final waste, spent fermentation liquor, can be disposed of with a minimal amount of treatment. For fed-batch cultivations, in which both of these substrates are used, the feed supply of both substrates has the advantage of being self-sterilizing and reduces the risk of contamination.

Uses of Alternative Carbon Sources

The advantages, if any, of using either acetic acid or ethanol as a fermentation feedstock instead of glucose should be seen as offering either improved productivity or overall reduction of costs. However, there is a further practical, commercial consideration: The use of these, and perhaps other, substrates may circumvent any patents on processes that have specified the use of glucose as a feedstock. If a process using an alternative feedstock also yields improvements in cellular composition, gives additional advantages for product recovery, or provides improved disposal methods for the waste processing streams from the fermentation, these will be extra incentives to switch substrates. Thus, the advantages or disadvantages of using an alternative feedstock are not exclusively associated with the material cost of producing the final product.

In dealing with both acetic acid and ethanol as substrates, one is immediately aware that, unlike glucose, these materials are toxic to most cells when used at concentrations higher than 1–2%. Since it is impractical to run a fermentation process with so little carbon substrate (the final cell biomass yields could not be more than 5

g/L and would in all probability be less than this), it is necessary in both cases to operate the fermentations as fed-batch cultures. In such processes, additional substrate is supplied to the fermentors at a rate designed to keep the prevailing concentration of the substrate below the level at which it would begin to inhibit growth. The question then becomes how to attain such a rate of substrate addition that, on the one hand, does not exceed the critical inhibitory concentration but, on the other hand, maintains a sufficient concentration of substrate that does not limit the growth rate. However, as shall be demonstrated in the following sections, this is not an insuperable task. Indeed, most industrial fermentation processes use some type of a fed-batch process mode to achieve optimal productivity; this indicates that there are many precedents that could be followed in using acetic acid or ethanol as substrates.

In order to run a successful fed-batch culture, it is usually necessary to monitor (directly or indirectly) the prevailing concentration of the key nutrient being supplied to the fermentor. Although devices for monitoring ethanol (or methanol) are now available, acetic acid is not easy to monitor directly on-line in a fermentor; its concentration has to be measured by some suitable indirect means. With this substrate, the authors were fortunate in being able to identify prior publications in which this problem had already been tackled and, importantly, resolved.

Use of Acetic Acid

Acetic acid is toxic to many microorganisms when the concentration is over about 5–10 g/L; few organisms, if any, will grow on it at over 20 g/L even at neutral pH values. Some initial and preliminary indication of acetic acid toxicity towards *C. cohnii* and other microalgae is given by Vazhappily and Chen (15) who reported that *C. cohnii* might be able to grow on acetate if its concentration was no more than 1 g/L; since the cultivation medium contained other utilizable carbon materials, this result is not entirely unambiguous. Some initial work in the authors' laboratories (M.E. de Swaaf, unpublished work) indicates that *C. cohnii* can grow on sodium acetate at 3 g/L, but this is accompanied by a sharp rise in pH that prevents good growth. This turns out to be the crucial problem in using acetate as feedstock.

When added to a fermentation medium, acetic acid has to be neutralized or least brought to the pH level used in the growth medium. This means addition of a counterion, usually Na^+ or K^+. When the cells start to grow, the acetate (CH_3COO^-) is consumed and is replaced by hydroxyl ions (OH^-) that, as a consequence, cause the pH to rise. In effect, NaOH is being produced by the consumption of the acetate ion. It is this rapid increase in pH that stops cells from growing. Consequently, if the rise in pH is to be avoided it has to be titrated with an acid; what better acid to use than acetic acid itself! Thus, a reservoir of acetic acid can be attached to the fermentor and linked to the pH meter and dosing pump; this equipment will then add acetic acid into the fermenatation medium on demand from the culture itself.

If a low concentration of acetic acid is used in the initial culture medium, the cells will begin to grow and consume the acetate; further acetic acid will then be pumped

into the fermentor to offset this rise in pH. The pH is controlled at its optimal value for growth. Consequently, the faster the organism grows, the faster will be the rate of acetic acid addition. Thus, the organism is always growing at its fastest possible rate. This concept was first enunciated by Martin and Kempfling (16), and the fermentation system was given the name "phauxostat." The name is derived from a device that can be used to maintain the pH of a culture (in this case *Escherichia coli*) by controlling the rate of substrate (glucose, glycerol, or lactic acid) addition into a fermenter based on the changes in the culture pH.

The first use of acetic acid in a phauxostat (or pH auxostat as it is now referred to) was, however, reported by Sowers *et al.* (17) who grew various acetotrophic, methane-producing bacteria on acetic acid and achieved an 18-fold increase in cell yield over previous levels. An application of this technique for SCO production was achieved by du Preez *et al.* (18) who showed that acetic acid was an excellent feedstock to produce an oil rich in γ-linolenic acid using *Mucor circinelloides* or *Mucor rouxii*. The reasons for the interest of this latter group in using acetic acid as a fermentation substrate rose from the availability of inexpensive acetic acid as a by-product from the Fischer-Tropsch synthesis process of manufacturing gasoline from coal, as carried out by Sasol Industries Ltd. of South Africa, and for which no satisfactory large-scale uses had been identified. Patents on this process were granted (19,20).

Thus, in spite of the uncertain growth of *C. cohnii* on acetate achieved in preliminary studies (15), it seemed that acetate could be used as a carbon source and that the application of a pH auxostat would then be an appropriate system to evalutate the possible use of this substrate to produce a SCO rich in DHA. *C. cohnii* was grown in a sea-salt/yeast extract medium with sodium acetate initially between 4 and 16 g/L; acetic acid (at 50%) was pumped in to maintain the pH at 6.5 (21). The best growth rate was achieved with 8 g acetate/L (Fig. 8.1). There was only a slight variation in the lipid content of the cells (45–50% of the biomass) with initial acetate concentrations up to 12 g/L and no real variation in the content of DHA in the oil (40–50%) at these concentrations. Since the cells needed to induce the synthesis of new enzymes in order to be able to use acetic acid (see Fig. 8.3), it was not surprising that more rapid growth was achieved using an inoculum grown on acetate than on glucose (21).

Overall, this system gives superior results over those obtained when glucose is used as a feedstock (8,9,21) producing as much as a 60% increase in the growth rate, a 70% increase in the lipid content of the cells (21), and a 50% increase in the level of DHA in TFA (Table 8.1). This increase is also reflected in the much higher level of DHA in the TAG fraction (i.e., edible oil fraction) of these cells. In other respects the composition of the other lipid components of extracted lipids is approximately equivalent between the two types of cells (21). The authors interpret this improved performance of *C. cohnii* on acetate compared to glucose as being at least partially due to its always growing at the maximum possible rate.

These findings have been confirmed by de Swaaf *et al.* (10) who, by using extended cultivation (up to 400 h), achieved final cell biomass yields of 109 g/L (Fig. 8.2). However, such high cell density cultures required vigorous mixing to sustain sufficient aerobic conditions; this transfer of oxygen into the system was complicated by increases in culture viscosity. The increase in viscosity was the result of the production of viscous extracellular polysaccharides (9). In large-scale industrial production of DHA, production of polysaccharides may cause problems, although the addition of a commercial polysaccharide-hydrolase preparation into the cultures can decrease the

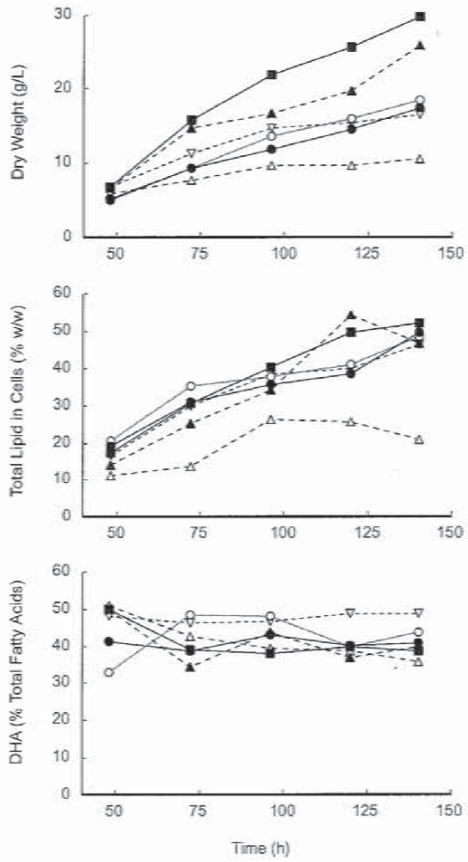


Fig. 8.1. (A) Growth, (B) lipid content of cells, and (C) docosahexaenoic acid (DHA) content of total fatty acids for *Crypthecodinium cohnii* grown at pH 6.5 in a pH auxostat culture with various concentrations of sodium acetate in the original medium and with additional acetic acid then being supplied into the culture on demand. Sodium acetate at (solid circle) 4, (open circle) 6, (solid square) 8, (solid triangle) 10, (inverted open triangle) 12, and (open triangle) 16 g/L. *Source:* Reference 21.

TABLE 8.1
Fatty Acid Profiles (as relative % w/w) of the Total Lipid and Neutral Lipids (e.g., Triacylglycerol) Fraction from *Crypthecodinium cohnii* ATCC 30772 Grown on Glucose in Batch Culture and Acetic Acid in a pH Auxostat Culture

	Glucose-Grown		Acetic Acid-Grown	
Fatty Acid	Total Lipid	Neutral Lipids[a]	Total Lipid	Neutral Lipids[a]
10:0	7	7	tr[b]	tr
12:0	24	19	7	8
14:0	26	22	21	16
16:0	8	9	18	16
16:1	1	2	2	2
18:0	1	1	2	1
18:1	7	9	11	10
22:6 (DHA)[c]	26	31	39	47

[a]Represents 74–75% of the total lipid in each case.
[b]tr = <0.5%
[c]Abbreviation: DHA, docosahexaenoic acid.
Source: Reference 21.

viscosity, and the stirring rate needed to maintain a fixed dissolved oxygen tension can then be decreased (10).

The overall conversion of acetic acid to biomass in the various pH auxostat cultures is calculated to be about 0.13 g biomass/g acetic acid used (10). This, though, is not a high conversion ratio. In part, it is due to the energy content of acetic acid being less than that of glucose (22). However, improvements in this biomass yield seem possible, since previous work with both yeasts and bacteria have achieved growth yields of over 0.35 g biomass/g acetic acid (23). Biomass yields for microorganisms grown on glucose, however, are normally between 0.45 and 0.5 g/g (23).

Another reason for this rather low growth yield by *C. cohnii* when using acetate may be attributed to the secretion of succinic acid in these cultures (Hopkins, S.A., unpublished work). The authors have also noted in the acetate pH auxostat cultures that the concentration of acetate in the fermentor did not remain constant with time but, in fact, gradually decreased from the usual starting value of 8 g acetate/L to less than 1 g/L in spite of the continuous addition of fresh acetic acid (Wynn, J.P., unpublished work). This suggests that there is a divergence of the acetate into some extracellular metabolite; this has been confirmed to be succinic acid accumulating at up to 5 g/L. This accumulation is attributed to the existence of a rate-limiting step in the conversion of succinic acid to fumaric acid as part of the tricarboxylic acid cycle (Fig. 8.3); this was exacerbated in acetate grown cells in which there are now two routes to produce succinate: one from isocitrate lyase and one from 2-oxoglutarate.

An empirical solution to this buildup of succinate, which is clearly a "waste" of acetate and accounts, at least in part, for the low biomass yield from acetate, is to add propionic acid at 1% (v/v) to the medium. Figure 8.4 shows the increased performance of the organisms under these conditions. There is a sustained increase in bio-

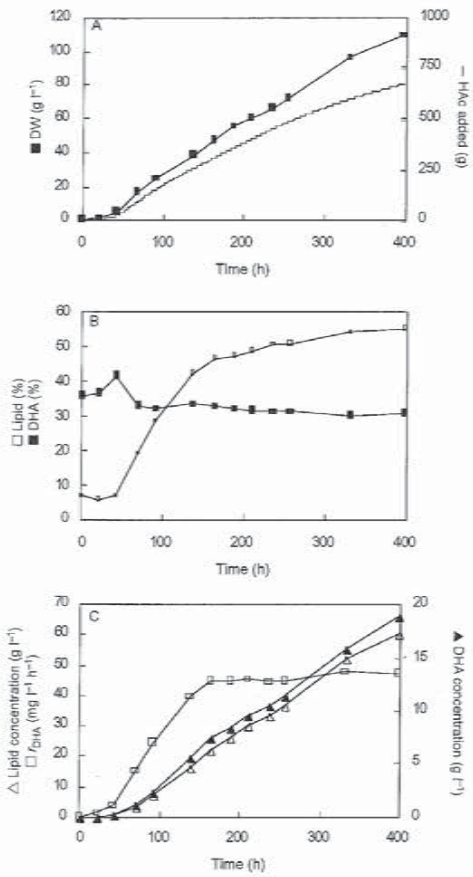


Fig. 8.2. Fed-batch cultivation of *C. cohnii* with a feed consisting of pure acetic acid. Initial medium: 10 g yeast extract/L, 25 g sea salt/L, 8 g NaAc/L; a 10% (v/v) inoculum was used. (A) Biomass dry weight (DW) and acetic acid added (HAc added), (B) Lipid content of dry biomass (lipid), DHA content of lipid (DHA). (C) Lipid concentration, overall volumetric productivity of DHA (r_{DHA}) and DHA concentration. *Source:* Reference 10.rDHA

mass to about 50 g/L and the total lipid now reaches 60% of the cell biomass by the end of growth. The amount of DHA in the lipid remained at approximately 35–40% TFA (Table 8.2).

The reason why propionate improves cellular growth when using acetate is not entirely clear, but it may be connected with propionate providing additional oxaloac-

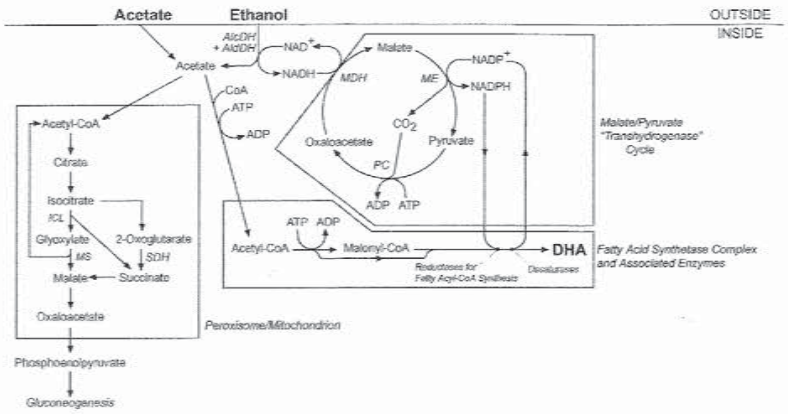


Fig. 8.3. Possible pathway for DHA biosynthesis from acetic acid and ethanol in *C. cohnii*. Ethanol in its conversion to acetate (acetyl-CoA) generates 2 mol of reduced nicotinamide adenince dinucleotide (NADH): one from the alcohol dehydrogenase (*AlcDH*) reaction and the other from the aldehyde dehydrogenase (*AldDH*) reaction. Further metabolism of acetate metabolism (from both ethanol and acetic acid) generates NADH in the mitochondrion and/or peroxisome *via* the reactions of the tricarboxylic acid. The conversion of acetate into biomass precursors (I.e., through gluconeogenesis) requires the synthesis of two enzymes to be induced: isocitrate lyase (*ICL*) and malate synthase (*MS*). Succinate is thus now generated by *ICL* and succinate dehydrogenase, (*SDH*) implying a metabolic imbalance in this organism and, as it accumulates in the culture medium, this implies that the rate of succinate utilization, by conversion to fumarate (not shown, but this is then converted to malate), is less than the combined rates of its synthesis. NADH is converted to reduced nicotinamide adenince dinucleotide phosphate (NADPH), which is needed for fatty acid synthesis and fatty acid desaturases, *via* a presumptive malate/pyruvate transhydrogenase cycle involving malic enzyme (*ME*), pyruvate carboxylase (*PC*), and malate dehydrogenase (*MDH*). This cycle has been demonstrated in other oleaginous microorganisms (30) but not yet in *C. cohnii*.

etate to the cells. This removes a metabolic bottleneck as indicated by the accumulation of succinate for the reasons given previously. The provision of oxaloacetate could come about *via* a transcarboxylase reaction that converts propionate into pyruvate and is well known in other microorganisms (24). The pyruvate, by the action of pyruvate carboxylase, would then provide additional oxaloacetate and other precursors needed to synthesize numerous cell components (23). Evidently, cells growing on acetate are not in metabolic balance; the addition of propionate then appears to improve this balance and consequently increases the efficiency of biomass production.

Contrary to expectations, the added propionate does not appear to be used to produce odd chain length FA in the lipid (Table 8.2). The overall FA profile in the

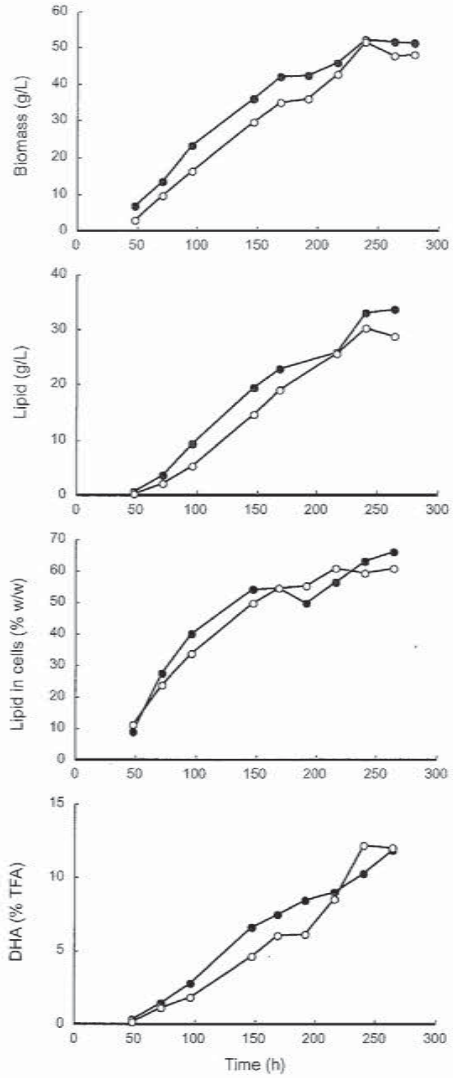


Fig. 8.4. Growth of *C. cohnii* ATCC 30772 in a pH auxostat culture using acetic acid as feedstock with the inclusion of 10 g propionic acid/L in the culture medium. The two curves given are from duplicate cultures run simultaneously in 5-L fermentors; (Kanagachandran, K., Anderson, A.J., and Ratledge, C., unpublished work).

TABLE 8.2
Analysis of Fatty Acids In Total Lipid from an Acetic Acid pH-Stat Culture of *C. cohnii* Grown in a Medium Containing 1% (v/v) Propionic Acid[a]

Fatty Acid	Fermentation Time (h)	
(Relative % w/w Total Fatty Acids)	96	240
10:0	0.96	2.8
12:0	6.7	8.1
14:0	19.4	13.8
14:1	—	~0.7[b]
15:0	tr[c]	0.1
16:0	20.5	14.2
16:1	~1[b]	~2[b]
17:0	0.03	0.05
18:0	1.4	0.05
18:1	14.9	15.4
19:0	—	0.02
20:0	0.4	0.25
22:1	0.14	0.3
22:6	34.1	39.9
Total Odd Chain Fatty Acids	0.03	0.17

[a]The course of fermentation is shown in Figure 8.4; only the results from two times of sampling (96 and 240 h) are shown here, but all other samples showed consistent values.
[b]Values that are estimated were not calculated by gas chromatography program.
[c]tr, trace = < 0.01%
(*Source:* Kanagachandran, K., Anderson, A.J., and Ratledge, C., unpublished work).

acetate-grown cells in which propionate is included in the medium does not show any trace of odd chain length FA, the characteristic product from this substrate, until the very end of growth. But even here, they do not constitute over 0.2% TFA. Normally one might have expected up to 10% TFA to be of odd chain length, having been derived from propionate being used by the FA-synthesizing enzymes. Thus, all of the added propionate is used in other aspects of the cells' metabolism; this clearly improves the performance in which acetate is the sole carbon source.

Ethanol as Carbon Source

Ethanol has been considered as a potential feedstock to produce SCO for some time with work being carried out in Czechoslovakia, the former USSR, and Japan in the 1970s and 1980s using several species of oleaginous yeast (24). Among these researches, a microcomputer-controlled, fed-batch fermentation was carried out by Yamauchi *et al.* (26) in which *Lipomyces starkeyi* was grown on ethanol maintained at its optimal concentration of 2.5 g/L. This technique allowed extremely high cell densities to be attained: 153 g/L over 140 h, with a cellular oil content in excess of 50%. There was an overall conversion coefficient of 0.23 g lipid/g ethanol used; this is in keeping with the very high theoretical conversion ratio of 0.54 g lipid/g ethanol

that has been calculated (25). For glucose, the theoretical conversion ratio would be about 0.32 (25).

With respect to DHA production in *C. cohnii*, the authors previously identified both the cultivation scale and volumetric productivity (r_{DHA}) as being major factors in determining the economic feasibility of this process (27). Factors that determine r_{DHA} include biomass concentration, lipid content of the cells, DHA content of the lipid, and cultivation time. Obviously, a high DHA content of the biomass is also desirable from the viewpoint of product recovery.

In laboratory-scale batch cultivations, the r_{DHA} for *C. cohnii* ATCC 30772 reported on glucose is 19 mg L^{-1} h^{-1} (8). Similar productivities were observed in fed-batch cultivations grown with a concentrated (50% w/v) glucose feed (10). In pH-controlled fed-batch cultivations with acetic acid as the carbon source, productivities of up to 48 mg DHA L^{-1} h^{-1} were achieved (Figs. 8.2, and 8.4) (7,10,11). This clearly indicates the strong impact that the carbon source can have on DHA productivity by *C. cohnii*.

Like acetic acid, ethanol could be a potential carbon source for *C. cohnii* provided that it is not toxic to the cells, can enter the cells, and can be metabolized. The ability to utilize ethanol suggests the presence of an alcohol dehydrogenase, to convert ethanol to acetaldehyde, and an acetaldehyde dehydrogenase to convert acetaldehyde to acetate (Fig. 8.3). As a carbon source for large-scale cultivation, ethanol may have advantages over acetic acid due to its lower cost and its ability to generate higher bio-

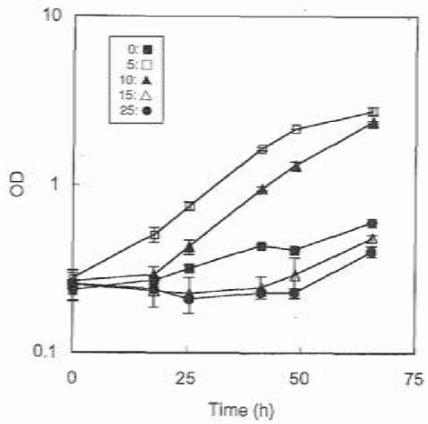


Fig. 8.5. Influence of initial ethanol concentration on growth of *C. cohnii* in shake-flask cultures. The medium contained: 2 g yeast extract/L; 25 g sea salt/L; a 10% (v/v) inoculum was used, and the medium was supplemented with ethanol at 0 (■), 5 (□), 10 (▲), 15 (△) or 25 (●) g L^{-1}. An optical density (OD) value of 1 corresponds to a cell dry wt value of approximately 1 g L^{-1}. *Source:* Reference 11.

mass yields per mol of carbon substrate (22,24). For practical reasons, due to its flammable nature, specific requirements will be necessary to store and add ethanol in large-scale applications. Furthermore, the application of ethanol as a fermentation feedstock requires equipment to control the ethanol concentration in a bioreactor at appropriate levels, as well as a controlled addition system.

The possible utilization of ethanol by cultures of *C. cohnii* was studied in initial shake-flask cultures. The alga was grown on a complex medium containing yeast extract, sea salt, and variable concentrations of ethanol (11). Growth when yeast extract was used as the sole carbon source was negligible. With ethanol at 5 or 10 g L^{-1}, cells were able to grow well (Fig. 8.5). The specific growth rates at these ethanol concentrations, calculated from the exponential part of the growth curves, was 0.05 h^{-1}. In contrast to cultures grown on 5 g ethanol L^{-1}, cultures grown on 10 g ethanol L^{-1} exhibited a significant lag phase. As ethanol concentrations reached 15 g L^{-1} and higher, growth of *C. cohnii* was substantially less. These data clearly demonstrate that ethanol, like acetic acid, cannot be used directly in batch cultures to achieve high biomass concentrations.

In order to avoid toxicity and provide sufficient ethanol for energy, growth, and metabolic activities, de Swaaf *et al.* (11) developed a process for ethanol fed-batch cultivations in a 2-L, computer-controlled laboratory bioreactor. As growth and lipid production require a sufficient supply of O_2, the dissolved O_2 tension (DOT) should preferably be kept over 30% air saturation; this can be accomplished by automatically controlling the stirrer speed (range: 200–1250 rpm) and by flushing with filter-sterilized air. Furthermore, since viscosity increases considerably in very high cell density cultures (10), a polysaccharase (Glucanex from Novo Nordisk, Neumatt, Switzerland) was added at 0.5 g/L to the cultivation broth.

The initial medium contained 5.5 g ethanol L^{-1} to provide growth from the start of inoculation. Compared to the acetic acid process described previously, ethanol addition cannot be controlled by changes in pH. Therefore, de Swaaf *et al.* (11) developed an automatic ethanol-feeding system based on changes in DOT. Similar results, however, have been obtained using a sterilizable ethanol sensor in the medium coupled to the ethanol-feeding system (Sijtsma, L., and van der Wal, H., unpublished work).

In the ethanol-grown fed-batch cultivation of *C. cohnii*, a total of 300 g ethanol was added into the fermentor over the 220-h fermentation time (Fig. 8.6A). The estimated specific growth rate over the first 52 h of incubation was 0.047 h^{-1}; this was in good agreement with the maximum specific growth rate estimated from ethanol-grown shake-flask cultures (Fig. 8.5).

Compared to glucose-grown cultures, a somewhat lower maximum growth rate was found. Between about 50 and 150 h, the biomass concentration increased linearly; this indicated that growth was being limited, probably by the supply and uptake of O_2. After this period, the increase in biomass concentration leveled off (Fig. 8.6). Over this time, the amount of lipid within the biomass increased from 9 to 35%; it reached a final value of 42% at the end of the fermentation process (Fig. 8.6). During

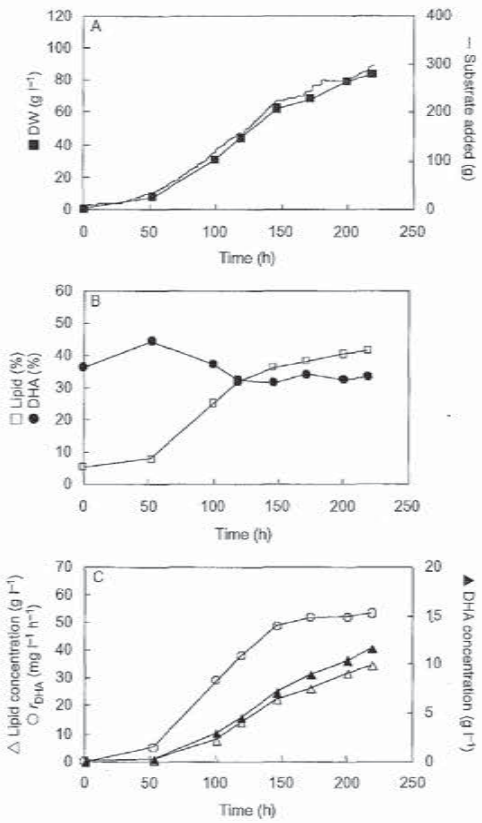


Fig. 8.6. Fed-batch cultivation of *C. cohnii* with a feed consisting of pure ethanol. (A) Biomass dry weight (DW) and ethanol addition. (B) Lipid content of dry biomass (Lipid), DHA content of lipid (DHA). (C) Lipid concentration, overall volumetric productivity of DHA (rDHA) and DHA concentration. The second, slightly higher, line indicates the total amount of ethanol added. *Source:* Reference 11.

the first 120 h of the process, the DHA content of the lipid varied between 44 and 32%. It remained constant at 33% during the final 100 h (Fig. 8.6). The final dry weight of cells, lipid, and DHA concentrations were 83, 35, and 11.7 g L^{-1}, respectively (Fig. 8.6). The latter value resulted in a volumetric DHA production rate (r_{DHA}) of 53 mg L^{-1} h^{-1}. The calculated biomass yield on ethanol was 0.31 g biomass/g ethanol used (11); this was about 2.4 times higher than the value calculated for acetic acid.

Lipid Production from C_2 Carbon Sources

Compared with glucose, the use of either acetic acid or ethanol as a carbon source resulted in a remarkable increase in DHA productivity by *C. cohnii* (Table 8.3). The major reasons for this improved productivity would appear to originate from both acetic acid and ethanol feeding directly into the pool of acetyl-CoA that is needed for lipogenesis (Fig. 8.3). The ability to maintain a high concentration of this key metabolite must, therefore, be crucial to lipid biosynthesis in *C. cohnii* and has been commented on elsewhere (27–29).

In glucose-grown cells, the main flux of carbon involves glucose uptake, glycolysis, transport of pyruvate into the mitochondrion, conversion of pyruvate into citrate, transport of citrate into the cytosol, and cleavage of citrate by adenosine triphosphate (ATP):citrate lyase to yield acetyl-CoA. This has been well explored in both yeasts and fungi (30); this then serves as a basis for understanding the possible processes for lipogenesis in other microorganisms.

If one assumes that a similar metabolic system operates in *C. cohnii* for DHA biosynthesis (and this may be a very big assumption), then one can see from Figure 8.3 that ethanol will produce higher yields of either cells or DHA (or both) than acetic acid. This is because ethanol, being more reduced than acetate, is able to generate more reducing power [in reduced nicotinamide adenince dinucleotide (NADH)] than acetate, and large amounts of this are needed to convert acetyl-CoA into FA. However, NADH is not used *per se* but needs to be converted by a "transhydrogenase" cycle involving malic enzyme, pyruvate carboxylase, and malate dehydrogenase (Fig. 8.3) into reduced nicotinamide adenince dinucleotide phosphate (NADPH); the same system is probably involved in providing NADPH for the various desaturases needed to produce DHA. In all, 26 mol of NADPH is needed for each mol of DHA that is synthesized: 2 for each of the 10 condensation reactions (used to reduce the β-ketoacyl and α,β-unsaturated fatty acyl intermediates), plus 1 for each of the six desaturases. Since each mol of ethanol produces 50% more NADH than each mol of acetate (31), it can be easily appreciated why ethanol is able to generate a higher biomass yield than acetate. NADH, beside being used for the biosynthesis of FA, is the principal source of energy (ATP) in the cells through its re-oxidation *via* the oxidative phosphorylation sequence (23). Thus, ethanol generates not only acetyl-CoA (as does acetate), but, more importantly, it produces more energy than acetate and thus gives higher yields of cells per unit weight.

Whether ethanol is used as a substrate for the large-scale production of DHA rather than acetic acid, or indeed in preference to glucose, will clearly have to await development of appropriate commercial interests. It may be relevant to point out that the conversion efficiency of ethanol to lipid is considerably higher than for both glucose and acetic acid (24,26) and thus may represent the most economical feedstock for SCO production.

TABLE 8.3
Comparison of Glucose (50% w/v), Acetic Acid pH Auxostat Culture and Ethanol Fed-Batch Cultures of *C. cohnii*

	Feed		
	Glucose	Acetic Acid	Ethanol
Time (h)	120	210	200
Biomass ($g \cdot L^{-1}$)	26	59	77
Lipid Content (% $w \cdot w^{-1}$)	15	50	41
Lipid Concentration ($g \cdot L^{-1}$)	3.8	30	31
DHA in Lipid (% $w \cdot w^{-1}$)	46	32	33
DHA Concentration ($g \cdot L^{-1}$)	1.7	9.5	10.4
Biomass Productivity ($mg \cdot L^{-1} \cdot h^{-1}$)	216	281	385
Lipid Productivity ($mg \cdot L^{-1} \cdot h^{-1}$)	31.6	143	155
DHA Productivity ($mg \cdot L^{-1} \cdot h^{-1}$)	14	45	52

Selected parameters are shown for time point 120 h (glucose), 200 h (ethanol), and 210 h (acetic acid).
Source: Reference 11.

Acknowledgments

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Chapter 9

Carotenoid Production Using Microorganisms

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Introduction

Carotenoids are biosynthesized by plants, algae, fungi, and bacteria (1,2). In these organisms they appear to play a number of roles, especially light-harvesting in plants and protecting cells from oxidative damage. A number of carotenoids, such as β-carotene, astaxanthin, and lycopene, are good antioxidants in lipid phases and function as free radical scavengers or singlet oxygen quenchers (3). Singlet oxygen ($^1O_2$) is known to damage DNA and to be mutagenic. Carotenoids act by absorbing the energy of the singlet oxygen onto the carotenoid chain; this leads to the degradation of the carotenoid molecule, but it prevents other molecules or tissues from being damaged. Carotenoids can also prevent the chain reaction production of free radicals initiated by the degradation of polyunsaturated fatty acids and thus prevent accelerated degradation of lipid membranes. It is these carotenoid properties that are used to explain epidemiological and experimental studies indicating that dietary carotenoids inhibit the onset of a range of diseases, such as cataracts, age-related macular degeneration, multiple sclerosis, arteriosclerosis, and some cancers (4–7).

Dietary carotenoids also play an important role in animal pigmentation and nutrition (8,9). The characteristic color of salmonid flesh is due mainly to astaxanthin and canthaxanthin (10). Similarly, the pigmentation of crustaceans, such as prawns and crabs, is due to astaxanthin. β-Carotene also has been shown to enhance fertility in cattle (11), pigs (8), and crustaceans (9).

"Natural" carotenoids used in human and animal nutrition are extracted from a number of natural plant sources; in the last 20 years several algal, fungal, and yeast sources have also been developed as commercial sources of β-carotene and astaxanthin. Microbial sources of other carotenoids, such as lycopene, lutein, zeaxanthin, and canthaxanthin, are also being studied.

β-Carotene

Dunaliella salina

The halophilic, unicellular, biflagellate green alga *Dunaliella salina* (Dunal) Teodoresco, also known as *Dunaliella bardawil*, is the richest natural source of the carotenoid β-carotene; it contains up to 14% of dry weight as β-carotene (12). The

β-carotene is accumulated as droplets in the algal chloroplast stroma, especially in high temperature, high salinity, high irradiance, and low nitrogen conditions (13–15). In *D. salina* the β-carotene occurs as a mixture of the *cis* (mainly 9-*cis*) and *trans* isomers. The formation of *cis* isomers is favored by low irradiances (16,17); however, there also appear to be some differences between strains (18). Commercial production commenced in Australia, Israel, and the U.S. in the 1980s; recently, *Dunaliella* production plants have also been established in India and Inner Mongolia (15,19,20).

D. salina is a photoautotroph, although it is able to utilize organic C, such as glycerol, to some extent in the light (21). The rate of carotenoid accumulation is a function of the total irradiance received by the cells (22,23).

All species of *Dunaliella* have a specific requirement for sodium and *D. salina* has the broadest reported NaCl tolerance range of any organism, from about 10 to 35% (w/v) NaCl (= saturation) (15). The optimum salinity for growth is about 22% NaCl, while the optimum salinity for carotenogenesis is over 30% NaCl (24). The actual salinity used for commercial culture is usually greater than 30% NaCl (i.e., about 10 times the salinity of seawater) because of the need to prevent the growth of predators, such as brine shrimp and the amoeba *Fabrea salina* (25), and avoid overgrowth by the non-carotenogenic *D. viridis* (26).

The optimum growth temperature for *D. salina* is between 21 and 40°C and is dependent on the strain and irradiance (27,28); however, the total growth range is from less than 0 to over 40°C (29,30). Higher temperatures stimulate carotenogenesis; the high temperatures (>35°C) and high irradiances experienced in the summer months at the *D. salina* production plant at Hutt Lagoon, Western Australia result in maximum β-carotene productivity at that time of year.

The solubility of CO_2 in the high salinity brines used to culture these algae is very low, and this is further reduced at the high temperatures and high pH normally encountered; the solubility of CO_2 at 15°C in 2 M NaCl is 1.02 while at 40°C it is only 0.53 (31). The addition of inorganic carbon either as CO_2 or HCO_3^- therefore stimulates growth, provided that there is no precipitation of carbonates (32).

The best nitrogen source for *D. salina* is nitrate; ammonium salts, urea, and nitrites are less effective (27,33). Care must be taken to control culture pH; nitrite uptake can lead to alkalinization, while ammonium uptake can lead to acidification of the medium, potentially leading to cell death. The optimum phosphate concentration for growth is approximately 0.02 $\mu g \cdot L^{-1}$ K_2HPO_4 and higher concentrations can inhibit growth (27).

Like other algae, *D. salina* requires Mg^{2+}, Ca^{2+}, K^+, chelated iron, and various trace elements (especially Mn, Zn, Co, and Cu) for growth. High sulphate concentrations above 2 mM are required for optimal cell division; higher concentrations also enhance carotenogenesis (34). No requirement for vitamins has been demonstrated, and the optimum pH for growth is between pH 7–9 (15).

Commercial production of *D. salina* for β-carotene is carried out either in extremely large (several hundred Ha in area), unmixed, open ponds as in Australia, or

more intensively in smaller paddle-wheel-mixed raceway ponds. Cultivation in closed systems, such as tubular photobioreactors, has also been attempted, but none of these systems has proven commercially viable as yet (26,35).

Open-pond culture can be likened to agriculture, with the cultures exposed to the daily and seasonal vicissitudes of weather (changes in temperature, light, rainfall, and wind) as well as potential contaminants from the environment. Cultures may be operated in either batch or continuous mode, with continuous culture being significantly more economical. A two-stage process, in which the algae are first grown at lower salinity to achieve maximum biomass followed by a high-salinity stage for maximum carotenoid accumulation has been considered, however this is uneconomical (12). An alternative two-stage culture process has been described by Ben-Amotz (36) in which the algae are first grown in a nitrate-rich medium to maximize biomass production and then diluted with a nitrate-deficient medium for the β-carotene accumulation stage. This culture regime increased β-carotene productivity by approximately 50%.

There is little published information on the actual productivity of commercial *D. salina* operations. Amotz (35) states that in 20-cm deep raceway ponds annual average productivities of about 200 mg β-carotene·m^{-2} · d^{-1} are achieved so that a 50,000 m^2 plant produces 10 kg β-carotene · d^{-1}. The average concentration of β-carotene in the cells is about 5% of dry wt, and the average concentration of algae in the ponds ranges from about 0.1 g dry wt · L^{-1} in unmixed ponds to up to 1.0 g · L^{-1} in raceway ponds.

Harvesting the algae and extracting and purifying the β-carotene represent major cost areas in the production of algal β-carotene. The exact details of the processes used by the various producers are therefore closely guarded. Harvesting *D. salina* is more difficult than most other microalgae. *D. salina* is a naked single cell, approximately 20 by 10 μm in size, neutrally or positively buoyant in a high specific gravity, high viscosity brine. Cell densities in the ponds are relatively low, between 0.1 to 0.5 g dry wt · L^{-1} and therefore very large volumes of medium have to be processed. These properties have led to the development of a range of harvesting methods or preconcentration methods to be used before more conventional harvesting methods. These include high-pressure filtration using diatomaceous earth as a filter medium (37), exploitation of salinity-dependent buoyancy properties in stationary and moving gradients (38), exploitation of the phototactic and gyrotactic responses of the algae (39,40), salinity-dependent hydrophobic adhesion properties of *Dunaliella* cells (41), and flocculation (42). Methods currently used in commercial production include centrifugation or flocculation and flotation.

Extraction of the β-carotene can be achieved using several methods. Conventional solvent extraction is efficient (43–45), however the presence of solvent residues in the final product is not acceptable for most markets. More acceptable extraction methods involve the use of hot vegetable oil (46) or supercritical carbon dioxide extraction (47,48). Alternatively, the algal biomass is drum-dried, stabilized, and sold as a β-carotene-rich nutritional supplement for human nutrition or as an additive for animal feed, such as prawn feed. Unlike the synthetic product, the algal product also contains small amounts of other carotenoids, such as α-carotene. The relative

proportion of the *cis* and *trans* isomers can be manipulated during the extraction and formulation process (49).

Recently, a new culture system has been described in which the cells are grown in a two-phase photobioreactor consisting of an aqueous and a biocompatible organic (dodecane) phase (50). In this system, the β-carotene is continuously removed ("milked") from the algal cells without impairing their viability, achieving β-carotene productivities of up to 2.45 mg·m^{-2}·d^{-1}. Although this productivity is 2–3 times higher than the productivity in current commercial open pond culture, it remains to be seen whether the process is commercially viable.

Blakeslea trispora

Several of the fungi of the order Mucorales synthesize β-carotene. These include *Phycomyces blakesleeanus, Choaneophora curcurbitarum*, and *Blakeslea trispora*. Industrial production of β-carotene from fungi has focused on *B. trispora* because of its inherently higher β-carotene content and the ability to grow well in liquid media in standard fermentations. Industrial production using *B. trispora* involves the separate culture of (+) and (–) mating strains followed by joint fermentation of the two strains in media rich in hydrocarbons (kerosene), carbohydrates, vegetable oils, and chemical additives (51,52). This process gives yields of 0.3% of dry wt β-carotene or about 3 g · L^{-1}. The process has been markedly improved over time with carotenoid contents of up to 20% of dry wt reported recently (53). β-Carotene is accumulated in the mycelium and synthesis is stimulated by trisporic acids, such as β-ionone, by a positive feedback control (54). In a mutant strain of *B. trispora* grown with β-ionone addition and with controlled oxygen addition and control of the age of the vegetative growth phase, biomass containing 30 mg β-carotene · g^{-1} dry wt can be produced in 5–6 d (55). Light has also been shown to stimulate carotenoid synthesis, although the degree of photo-induction of carotenogenesis varies with light wavelength and the length of exposure time (54). Jeong *et al.* (56) have postulated that this effect of light might be by oxidative stress through the production of free oxygen radicals and they have shown that hydrogen peroxide increases β-carotene production.

Blakeslea is unusual among the Mucorales in that carotene biosynthesis is not inhibited by the end product β-carotene as is the case in *P. blakesleeanus* (57,58). *Blakeslea* and *Phycomyces* also differ in their response to blue light and many chemicals (54,59) and, in *Phycomyces*, the highest carotenoid contents are achieved on solid substrates or in quiet (unmixed) liquid media.

Astaxanthin

Haematococcus pluvialis

The freshwater green flagellate alga, *Haematococcus pluvialis* (*H. lacustris* is also *H. pluvialis*), can accumulate up to about 6% (about 2–4% in commercial cultures) of dry weight as astaxanthin, making it the best natural source of this carotenoid. In nature

and in culture, the alga exists in three cell forms: a motile, naked, biflagellate stage; a non-motile, naked palmella stage; and a non-motile, thick-walled aplanospore stage. The astaxanthin is accumulated in droplets in the perinuclear cytoplasm in the aplanospore stage of the life cycle. The astaxanthin occurs as the 3S, 3′S isomer in the form of free astaxanthin as well as mono- and di-esters with $C_{16:0}$, $C_{18:0}$, $C_{20:0}$, and $C_{18:1}$ fatty acids (60). Commercial production commenced in the mid-1990s in the US (Hawaii) and Sweden, and later in Israel and India.

The optimum growth temperature is between 15–20°C and in batch culture, the motile stage predominates during the logarithmic phase of growth and the carotenogenic aplanospores are formed during the stationary phase. This change in cell morphology from rapidly dividing, shear-sensitive, flagellated cells to the extremely robust, non-motile aplanospores generally means that a two-stage batch growth process is required. In this process conditions are first optimized to grow the non-carotenogenic flagellate cells to achieve the maximum biomass; this is followed by a second stage in which the cells are "stressed" to induce aplanospore formation and maximize astaxanthin accumulation. *H. pluvialis* can grow photoautotrophically as well as mixotrophically or heterotrophically using organic C sources, such as acetate (61–63); however, heterotrophically grown cells grow more slowly and produce only small amounts of astaxanthin in the dark (64). In mixotrophic growth, acetate and pyruvate addition enhances growth rate, cell yield, and astaxanthin formation (62,65). However, there is significant variability between strains in their response to acetate and their ability to use other carbon sources, such as glucose, glycerol, and glycine (66). The maximum astaxanthin content of the cells is also strain dependent (67). Strain selection is therefore an important factor in the development of a commercial process for this alga. Increased astaxanthin production in several strains has also been achieved through mutagenesis (68), but better results have been obtained through the isolation and screening of new strains (Borowitzka, M., unpublished results).

Carotenogenesis is stimulated by a wide range of "stress" factors, such as nutrient limitation, especially N-limitation, high light, high temperatures, or NaCl addition (62,69–71)—in fact, almost any factor that inhibits growth. Boussiba and Vonshak (69) were able to demonstrate that vinblastin addition inhibited cell division and promoted astaxanthin accumulation. Other studies also have shown that reactive oxygen species enhance astaxanthin synthesis by an, as yet, unknown mechanism (72–74). The light regulation of carotenogenesis in *Haematococcus* is apparently under photosynthetic redox control. The plastoquinone pool appears to function as a redox sensor and reduction of the plastoquinone pool leads to the transcriptional activation of most, if not all, genes involved in astaxanthin biosynthesis (75).

Current commercial processes first produce the algal biomass in closed photobioreactors, such as bubble columns, plate reactors, or tubular photobioreactors, under optimal conditions in a nutrient-replete medium. This is then followed by a carotenoid-accumulation step under reduced N and P conditions in a high light environment either in raceway ponds or in tubular photobioreactors. This whole growth

cycle takes several weeks (76,77). The final product is a spray-dried powder of astaxanthin-rich aplanospores that has been mechanically or enzymatically broken and stabilized; it can be used as a pigmenter for salmonid fish (78,79) and as a human nutritional supplement (80).

Phaffia rhodozyma/Xenophyllomyces dendrorhous

The basidomycetous yeast, *Phaffia rhodozyma/Xenophyllomyces dendrorhous* is unusual among carotenoid-containing yeasts in that it has the ability to vigorously ferment sugars. On the basis of life history studies and sequence analysis of rDNA IGS and ITS regions, there appear to be the distinct anamorphic (asexual) species *P. rhodozyma* and the teleomorphic (producing both sexual and asexual spores) *X. dendrorhous* (81,82). This yeast produces unesterified astaxanthin, predominantly in the 3*R*,3′*R* form, and has been extensively studied as a commercial source of this carotenoid because of the very high cell densities of >50 g dry wt·L^{-1} when grown in a fermentor (83). Cell yield and carotenoid production decrease at high sugar concentrations, therefore fed-batch fermentation is used to maximize productivity (84).

The main limitations to commercial utilization are the low levels of astaxanthin found in wild-type isolates and the thick wall and capsule of the yeast that markedly reduces the bioavailability of the astaxanthin. However, several companies have developed astaxanthin-hyperproducing strains that produce >1% of dry wt astaxanthin by chemical mutagenesis and screening; genetic engineering methods are also under development (85–88). An (89) has improved growth and carotenogenesis in the carotenoid-hyperproducing mutant strain 2A2N in media containing tricarboxylic acid cycle intermediates. This suggests that the use of media containing molasses (90), corn steep liquor (91), or corn wet-milling co-products (92) can be used in conjunction with grape juice (93) as a source of TCA cycle intermediates to increase biomass production with a high carotenoid content. A range of mechanical, enzymatic, and chemical methods have also been developed to break the cell wall of the yeast and improve the bioavailability of the astaxanthine (94,95).

Other Carotenoids and Other Organisms

Carotenogenesis is widespread among microorganisms, and a number of species are being studied as potential new industrial sources of carotenoids. In addition, cloning of carotenoid pathways provides a potential tool to engineer overproducing strains or create carotenoid producers from non-producing species (96).

Many species of green algae other than *H. pluvialis* are known to produce high concentrations of astaxanthin as their major carotenoid. These include *Chlorococcum* sp., *Neochloris wimmeri*, *Chlorella zofingiensis*, and *Protosiphon botryoides* (Table 9.1). Potential algal sources of lutein include the green algae *Muriellopsis* sp. and *Chlorococcum citrifome* (102). A mutant of *D. salina* that produces up to 6 mg·g^{-1} zeaxanthin has also been isolated (111). The yeast *Rhodotorula glutinis* produces the carotenoids torulene, torularhodin, γ-carotene, and β-carotene in various proportions.

TABLE 9.1
Carotenoid Content and Yield for a Selected Range of Microorganisms[a]

Organism	Principal Carotenoid(s) Accumulated	Maximum Carotenoid Content Reported(mg·g^{-1} dry wt)	Carotenoid Yield (mg·L^{-1})	References
Algae				
Dunaliella salina	β-Carotene	~500 (–1400)	~5–50	15
Haematococcus pluvialis	(3*S*,3′*S*) Astaxanthin	~300 (–600)		77,97
Chlorococcum sp.	Astaxanthin (Canthaxanthin) Adonirubin, 4′-Hydroxy Echinenone	8	45	98–100
Neochloris wimmeri	Astaxanthin	19.3		101
Protosiphon botryoides	Astaxanthin	14.3		101
Chlorella zofingiensis	Astaxanthin	6.8	24.8	101,102
Trentepohlia aurea	β-Carotene	21		103
Chlorella sorokiniana	Lutein (β-Carotene)	4.3	730	104
Chlorella protothecoides	Lutein	6.5	136	105
Muriellopsis sp.	Lutein	5.5	35	102
Chlorococcum citriforme	Lutein	7.4	38	102
Fungi and Yeasts				
Xanthophyllomyces dendrorhous/Phaffia rhodozyma	(3*R*,3′*R*) Astaxanthin	>10	~5	106
Blakeslea trispora	β-Carotene	17		59
	Lycopene	41		107
Phycomyces blakesleeanus	β-Carotene			
Mycobacterium aurum (mutant)	Lycopene	7.4	16.2	108
Bacteria				
Brevibacterium KY-4313	Canthaxanthin	0.6	1–2	109,110

[a]Some of the data have been recalculated from the information supplied in the sources.

Bhosale and Gadre (112) have isolated a β-carotene-overproducing mutant of this yeast that contains up to 2 mg·g^{-1} β-carotene (82% of the total carotenoids). The food yeast *Candida albicans* has also been genetically engineered to produce up to 7.8 mg·g^{-1} lutein (113). Recently, a new bacterial genus that contains about 0.13 mg·g^{-1} (3*S*, 3′*S*)-astaxanthin has been isolated (114). Ninet and Renaud (52) report yields of 10-40 mg·L^{-1} of zeaxanthin in a *Flavobacterium* sp. and yields of up to 0.9 g·L^{-1} of mostly unidentified xanthophylls with isorenieratene (an aromatic carotene) predominating in the actinomycete *Streptomyces mediolani*. The potential of these nonphotosynthetic bacteria as commercial carotenoid sources remains to be fully explored.

Conclusion

Several microbial species have been successfully commercialized in the last 20 years as natural sources of the carotenoids β-carotene and astaxanthin. New sources of these carotenoids, as well as lutein and lycopene, are in advanced stages of development. Genetic engineering is also providing a powerful tool for understanding the carotenoid biosynthetic pathways and their control; this information can be used to optimize production systems. Genetic engineering has also been used to create overproducing strains and to transform non-carotenogenic species into carotenoid producers. At this stage, this is of limited commercial interest because the natural carotenoid market is very averse to any genetically modified organism product. However, this may change in the future, and it is clear that commercial carotenoid biosynthesis using microorganisms has a great and colorful future.

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Chapter 10

Prospects for Eicosapentaenoic Acid Production Using Microorganisms

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Introduction

The therapeutic significance of n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), has been clearly indicated by recent clinical and epidemiological studies (1,2). EPA performs many vital functions in biological membranes and serves as a precursor of a variety of lipid regulators in cellular metabolism; as a result, it plays an important role in the treatment of various human diseases, such as rheumatoid arthritis, heart disease, cancers, schizophrenia, and bipolar disorder (3–8). These findings have led to considerable interest in developing commercial processes to produce EPA (9,10).

Marine fish oil is presently the richest source of EPA and is currently used for its commercial production; however, EPA recovery and purification from fish oils is expensive (9). Microorganisms, including microalgae, lower fungi, and marine bacteria, are the primary producers of EPA; fish usually obtain EPA via bioaccumulation in the food chain (11,12). Much effort is being devoted to develop a commercially feasible technology to produce EPA directly from microorganisms. The aim of this chapter is to review the recent advances in EPA production by microorganisms, in particular by microalgae.

EPA

Structure and Significance

EPA is an important n-3 PUFA. Another important and related n-3 PUFA is DHA. The chemical structures of EPA and DHA are shown in Figure 10.1. In living cells, EPA and DHA are normally esterified to form complex lipids. EPA plays an important role in higher animals and humans as a precursor of a group of eicosanoids that are crucial in regulating developmental and regulatory physiology. The eicosanoids are hormone-like substances including prostaglandins, thromboxanes, and leukotrienes (LT). Arachidonic acid (AA, 20:4 n-6) and EPA are precursors of eicosanoid compounds (Fig. 10.2). However, the eicosanoids from these two fatty acids (FA) are different both



Fig. 10.1. Chemical structure of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). *Source:* Reference 111, with permission from Elsevier.

structurally and functionally, and are sometimes even antagonistic in their effects. A balanced uptake of EPA/AA can prevent eicosanoid dysfunctions and may be effective in treating a number of illnesses and metabolic disorders (3,4).

EPA is a potential anti-inflammatory agent (14,15). One possible mechanism is that EPA-derived eicosanoids compete with AA-derived eicosanoids by forming LT_5, and, hence, reducing the level of LT_4 in rheumatoid arthritis patients (Fig. 10.2). n-6

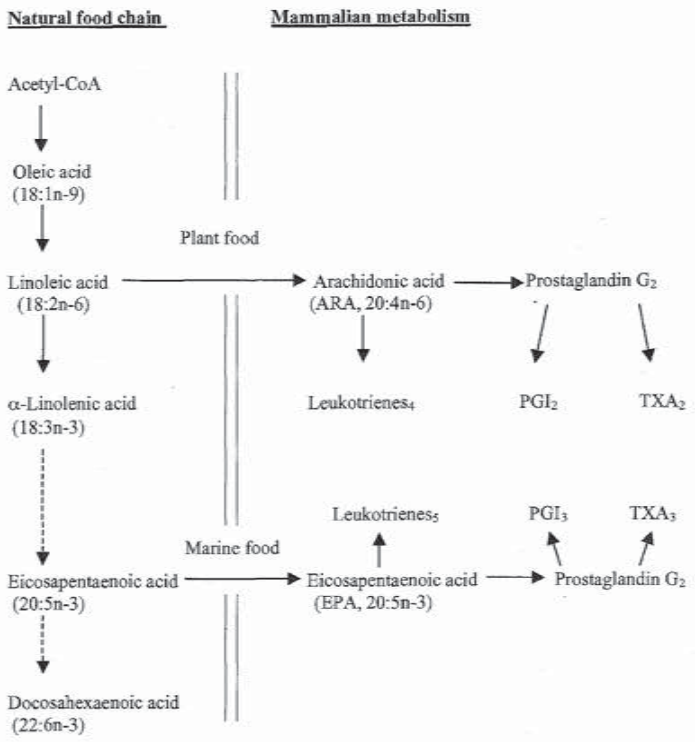


Fig. 10.2. Metabolic pathways of n-3 and n-6 eicosanoids from arachidonic acid (AA) and eicosapentaenoic acid (EPA). *Source:* Reference 13.

Eicosanoids have a strong effect on body tissues while n-3 eicosanoids possess a different or weaker potency with respect to various cellular responses (16). EPA also possesses therapeutic activity against cardiovascular diseases. For example, EPA can prevent atherosclerosis by decreasing the level of low-density lipoproteins (17). EPA appears to affect the electrical behavior, rhythms, and chemical responses of the heart; therefore, it reduces the likelihood of heart attack and arrhythmias (abnormalities of the heartbeat). EPA is capable of reducing the tendency toward thrombosis by reducing the level of fibrinogen, an activation factor in the occurrence of thrombosis (18). Recently, clinical evidence has shown that EPA plays a significant role in the treatment of schizophrenia and bipolar disorder by increasing the n-3 FA content of erythrocyte membrane and plasma (5–8).

Biosynthesis

The biosynthesis of EPA occurs through a series of reactions that can be divided into two distinct steps. First is the *de novo* synthesis of oleic acid (OA, 18:1n-9) from acetate. This is followed by conversion of OA to linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), and a number of subsequent stepwise desaturation and elongation steps to form an n-3 PUFA family that includes EPA (Fig. 10.3).

Almost all biological systems, including microorganisms, insects, higher plants, and animals, are capable of *de novo* FA synthesis from acetate to FA, with OA as the major product. The biosynthesis starts with the carboxylation of acetyl-CoA. Acetyl-CoA is synthesized from acetate or pyruvate by the action of glycolytic enzymes, and then converted into malonyl-CoA; this is used to drive a condensation reaction to extend the acyl group to stearic acid (18:0). This is desaturated to OA that, in turn, is desaturated by a Δ12 desaturase to form LA, and then a final Δ15 desaturase forms ALA. The n-9, n-6, and n-3 FA families are formed from these precursors by a series of desaturation and elongation reactions. The biosynthesis of the three FA families is shown in Figure 10.3. The three parent FA—OA, LA, and ALA—compete with each other for the Δ6 desaturase. The affinity of this enzyme to the substrate and the amount of substrate available determine which metabolic pathway is predominant (19). Generally, the first Δ6 desaturation is the limiting step, and ALA has the highest affinity for Δ6 desaturase, followed by LA and OA.

Most algae, fungi, bacteria, mosses, insects, and some invertebrates possess the desaturase and elongase required to synthesize various PUFA. They are the primary producers of these FA in nature (20–23). In contrast, higher plants and animals lack the requisite enzymes and thus, rarely produce PUFA above C_{18} (4).

Sources

Fish Oils. The annual worldwide demand for EPA is approximately 300 T (24). Fish oil is the conventional source of EPA, but there are a number of disadvantages in using it to produce a pharmaceutical/clinical grade material.

The quality of fish oil depends on fish species, season, and geographical location of the catching sites. Marine fish oil is a complex mixture of FA with varying chain

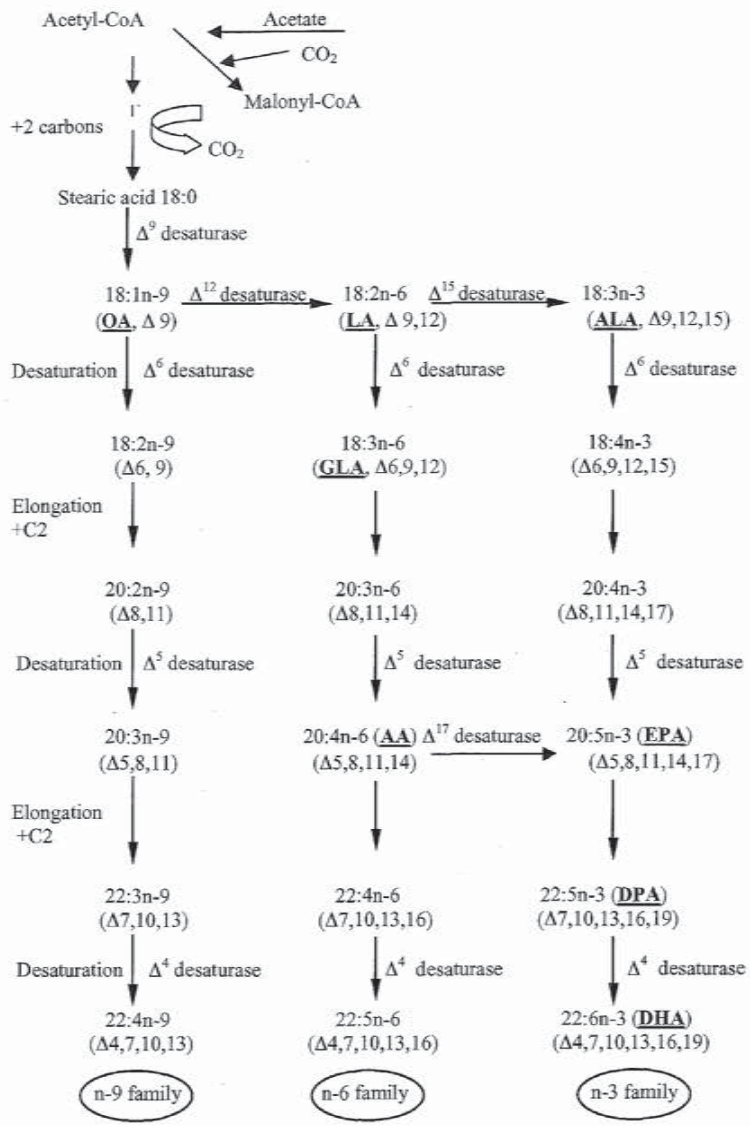


Fig. 10.3. Biosynthesis of polyunsaturated fatty acids. Abbreviations: α-linolenic acid, ALA; γ-linolenic acid, GLA; arachidonic acid, AA; docosapentaenoic acid, DPA; and linoleic acid, LA; oleic acid, OA. *Source:* Reference 111, with permission from Elsevier.

lengths and degrees of unsaturation. For clinical applications, however, it is necessary to produce an oil containing EPA as the sole PUFA. Even an oil containing a mixture of only EPA/DHA or EPA/AA is not satisfactory for clinical applications because of their different pharmaceutical functions, not to mention the complexity of lipid and FA of fish oil. Moreover, EPA purification from low-grade fish oil requires a number of physical steps, including the use of large-scale preparative high-performance liquid chromatography (HPLC), that are prohibitively expensive (9). In addition, fish oil has its peculiar taste and odor (12). Marine fish stocks are also subject to seasonal and climatic variations (4) and, in some locations, are physically dwindling due to chronic over-fishing. Finally, but by no means least, is the content of deleterious man-made chemicals and organo-heavy metals, including mercury compounds, in fish oils. These materials have been ingested by the fish and then concentrated in their livers; these are the principal organ used to obtain oils. All these factors argue against the continued use of fish oils as a supply of EPA and DHA, while encouraging the use of alternative sources that, at the present time, has to be microbial.

Microorganisms. A variety of phylogenetically and physiologically diverse bacteria isolated from cold marine environments produce EPA as a part of their normal metabolism (11,25–32). The marine bacterium, *Shewanella*, has been widely studied for its EPA content (Table 10.1). Other EPA-producing bacterial genera include *Alteromonas*, *Flexibacter*, *Psychroflexus*, and *Vibrio* (25,27,30,32,33). The bacterial synthesis of FA, like EPA, can be affected by varying culture conditions, such as temperature, and carbon and energy sources, as well as bacterial growth phase (32). One apparent disadvantage for bacterial EPA production is that EPA predominates in total fatty acid (TFA) only when bacteria grow at very low temperature and high pressure (30–33); consequently, it is difficult to exploit these bacteria for commercial production of EPA.

Many lower fungi belonging to the genera *Mortierella*, *Pythium*, and *Saprolegnia* are capable of producing considerable amounts of EPA (34–40). The fungus *Mortierella alpina* has been widely investigated for its potential of EPA production (Table 10.1, see Chapter 2). Temperature is crucial in regulating the formation of different FA in this fungus. It produces AA exclusively at very high levels if the temperature is over 20°C (see Chapter 5). At low temperature (12°C), however, this fungus accumulates large amounts of EPA (39). The conversion of AA to EPA at lower temperature is considered to be catalyzed by the Δ17 desaturase (Fig. 10.3, 33). Exogenous addition of ALA to cultures of *M. alpina* can also lead to the enhancement of EPA yield (35). Similar results have been reported with *Mortierella elongata* NRRL 5513 (40).

Among the various microorganisms, microalgae are the most abundant source of EPA. EPA has been found in a wide variety of marine microalgae, including in the classes Bacillariophyceae (diatoms), Chlorophyceae, Chrysophyceae, Cryptophyceae, Eustigamatophyceae, and Prasinophyceae (Tables 10.2 and 10.3). A study of *Porphyridium cruentum* by Cohen (53) has established the production potential of EPA by this microalga (see Chapter 4). Ohta *et al.* (54) has also reported

TABLE 10.1
Proportions of PUFA in Bacteria and Fungi

	PUFA (% TFA)			
Organisms	20:4 AA	20:5 EPA	Cultivation/Isolation Method	Reference
Bacteria				
Shewanella benthica	—	16	Intestine of holothurian, South Atlantic Ocean, depth 4575 m, 4°C	26
Shewanella woodyi	—	16	Detritue, Alboran Sea, depth 370 m, 25°C	26
Shewanella sp.	1.9	6.5	Antarctic seawater, 4°C, 30 d	31
Shewanella marinintestina	1.1	17.5	Squid body, Yokohama, Japan, 20°C	28
Shewanella schlegeliana	2.2	18.6	Black porgy intestine, 20°C	28
Shewanella sairae	0.7	15.2	Saury intestine, Pacific Ocean, 20°C	28
Shewanella pealeana	1.6	19.1	Marine broth, 20°C, 2 d	28
Shewanella hanedai	1.4	23.1	Marine broth, 15°C, 2 d	28
Shewanella gelidimarina	0.7	14.0	Marine broth, 15°C, 2 d	28
Fungi				
Mortierella alpina 20-17	22.9	4.9	GY medium, 28°C, 6 d	33
Mortierella alpina 1-83	24.2	19.8	GY medium, 12°C, 7 d	34
Mortierella alpina 2O-17	38.7	17.1	GY medium, 12°C, 7 d	34
*Mortierella alpina*1S-4	28.4	13.9	GY medium, 12°C, 7 d	34
Mortierella parvispora	14.4	10.9	YM medium, 12°C, 7 d	34
Mortierella hygrophila	13.6	10.4	GY medium, 12°C, 7 d	34
Saprolegnia sp. 28YTF-1	1.0	3.6	PYM medium, 28°C, 7 d	35
Pythium ultimum	6.1	8.2	Solid substrate (linseed oil, barley), 2°C	36
Pythium irregulare	24	27	1% glucose (basal medium), 24°C, 8 d	37

Abbreviations: PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; AA, arachidonic acid; TFA, total fatty acids.

a high proportion of EPA in *Porphyridium purpureum*. Most diatoms have a high content of EPA (Table 10.3). The biotechnological potential of diatoms has been reviewed recently by Lebeau and Robert (55). The diatoms *Phaeodactylum tricornutum* and *Nitzschia laevis* have been intensively investigated for their EPA production potential (54,56–58). In December 2002, the marine diatom *Odontella aurita* was filed to the Advisory Committee on Novel Foods and Processes of the European Union (EU) to market the alga as novel food (59). The alga is reported to be rich in EPA, as well as in essential trace elements, and can be grown in simple seawater or salt groundwater in outdoor tanks. The cultivation process of *O. aurita* has been favorably commented upon by the French Competent Authority, and it is under review by other member states of the EU. In contrast to a large number of EPA-containing microalgae that are known, only a few microalgal species have clearly demonstrated industrial production potential (Tables 10.2 and 10.3). This is due primarily to the low specific growth rate and low cell density of the microalgae grown under conventional photoautotrophic conditions. In open pond systems, for example, a typical cell density was just 0.5 $g \cdot L^{-1}$ (60).

TABLE 10.2
Proportions of PUFA in Marine Microalgae[a]

Organisms	PUFA (% TFA) 20:4 AA	20:5 EPA	22:6 DHA	Reference
Chrysophyceae				
Monochrysis lutheri	1	19	—	41
Pseudopedinella sp.	1	27	—	41
Coccolithus huxleyi	1	17	—	41
Cricosphaera carterae	3	20	—	41
Cricosphaera elongata	2	28	—	41
Isochrysis galbana	—	15	7.5	42
Eustigmatophyceae				
Monodus subterraneus	4.7	33	—	43
Nannochloropsis sp.	—	35	—	44
Nannochloris sp.	—	27	—	41
Nannochloris salina	1	15	—	41
Chlorophyceae				
Chlorella minutissima	5.7	45	—	45
Prasinophyceae				
Hetermastrix rotundra	1	28	7	41
Cryptophyceae				
Chromonas sp.	—	12.0	6.6	46
Cryptomonas maculata	2	17	—	41
Cryptomonas sp.	—	16	10	41
Rhodomonas sp.	—	8.7	4.6	46

[a]All algae cultures were grown photoautotrophically.
DHA, dococahexaenoic acid. For other abbreviations, see Table 10.1.
Source: Reference 111, with permission from Elsevier.

EPA Production by Microalgae

To date, microalgae have been seen as promising candidates for commercial production of EPA because of the following traits: Most microalgae have a high cellular EPA content. Microalgae require relatively simple nutrients and mild environmental factors. Their cultivation conditions are easy to control. Some microalgae are capable of heterotrophic growth; this offers the possibility of greatly increasing cell density and productivity by using high cell-density culture techniques.

Factors Influencing EPA Production

Culture Age. Oleaginous microalgae tend to store their energy source in the form of lipids as the culture ages. In contrast, the cellular content of PUFA (including EPA) tends to follow a sigmoid curve; the PUFA content increases until the culture approaches the late growth or early stationary phase of growth and then decreases gradually at the late stationary and death phases [(41), see Chapter 4]. In heterotrophic cultures of the marine diatom *N. laevis*, the cellular content of EPA increases as the culture ages, but the proportion of EPA (as %TFA) remains relatively constant (61).

TABLE 10.3
PUFA Compositions of Diatoms

Organisms	PUFA (% TFA) 20:4 AA	20:5 EPA	22:6 DHA	Growth Mode	Reference
Asterionella japonica	11	20	—	Photoautotrophic	41
Amphora coffeaformis	4.9	1.4	0.3	Photoautotrophic	46
Biddulphia sinensis	—	24	1	Photoautotrophic	41
Chaetoceros sp.	3.0	16.7	0.8	Photoautotrophic	46
Cylindrotheca fusiformis	—	18.8	—	Heterotrophic	47
Fragilaria pinnata	8.7	6.8	1	Photoautotrophic	46
Navicula incerta	—	25.2	—	Heterotrophic	47
Navicula pelliculosa	—	9.4	—	Heterotrophic	47
Navicula saprophila	2.7	16.0	—	Mixotrophic[a]	48
Nitzschia closterium	—	15.2	—	Photoautotrophic	49
Nitzschia frustulum	—	23.1	—	Photoautotrophic	49
Nitzschia laevis	6.2	19.1	—	Heterotrophic	50
Phaeodactylum tricornutum	—	34.5	—	Photoautotrophic	51
Skeletonema costatum	—	29.2	—	Photoautotrophic	52

[a]The algal cells were grown in the light with acetate as carbon source.
For abbreviations see Tables 10.1 and 10.2.
Source: Reference 111, with permission from Elsevier.

Nutritional Factors. Several major nutrients are necessary for microalgal growth, including carbon, nitrogen, and phosphorus sources. Diatoms also require silicon. In addition, all microalgae require various salts, such as sodium, potassium, calcium, and trace elements (Fe^{3+}, Cu^{2+}, Co^{2+}, and Zn^{2+}) to maintain a natural marine environment. Vitamin B_{12} and biotin are also required in many cases.

Carbon sources are necessary to provide the energy and carbon skeletons for cell growth. Under photoautotrophic conditions, algae use CO_2 as their carbon source. Heterotrophic microalgae, however, are capable of growing in darkness; therefore, they must derive energy from at least one organic carbon source, which is often provided in the form of acetate or glucose (62–64). Other carbon sources, including mono-, di-, and polysaccharides (such as fructose, sucrose, lactose, and starch) may also be usable by some species. Some obligate phototrophic algae can be converted into heterotrophs by genetic engineering and are capable of utilizing organic carbon sources (65). Vegetable oils, such as linseed, corn, and canola, may promote growth and/or EPA production, depending on the microalgal species used. Generally, the C/N ratio of the medium may influence the final cellular lipid content by controlling the switch between protein and lipid syntheses (66). A high C/N ratio favors lipid accumulation; this is triggered by nitrogen depletion in the culture (67). However, Chen and Johns (68) found that a high C/N ratio led to a lower proportion of unsaturated FA in the microalga *Chlorella sorokiniana*. The reason might be that under nitrogen shortage, triacylglycerols (that are mostly PUFA-poor) are accumulated. Subsequently, the share of PUFA-rich polar lipids decreases.

Simple nitrogen sources, such as nitrate and urea, can support the growth and EPA production in the microalgae *P. tricornutum* (51) and *N. laevis* (61). When using ammonium as the nitrogen source, the pH of the medium tends to fluctuate markedly; this ultimately inhibits the growth and EPA production by the diatom (61). For example, if an ammonium salt, such as its sulphate or chloride is used, the assimilation of the ammonium ion leaves either sulphuric or hydrochloric acid behind. These are then the reason for the pH change and loss of cell viability. Complex nitrogen sources, such as yeast extract, tryptone, and corn steep liquor, can promote the growth of most microalgal species by providing amino acids, vitamins, and growth factors (61,69). Because their concentrations are usually very low, the contribution to cost of such complex nitrogen is not significant compared to the carbon source used.

Phosphate plays an important role in the energy transfer of the cells. The syntheses of phospholipids and nucleic acids also involve phosphate. Because n-3 PUFA in the cells mainly exist in the form of polar lipids, such as phospholipids (70), the phosphate level may significantly affect the cellular content of n-3 PUFA. Yongmanitchai and Ward (51) investigated the effect of phosphate concentrations on EPA production of *P. tricornutum* and found that EPA yield was higher at a higher phosphate concentration. However, in a culture of *Pythium irregulare*, a high initial phosphate concentration lowered the EPA yield (71). Ohta *et al.* (54) found the optimal phosphate concentration for EPA production in *Porphyridium purpureum* to be 3 mM (in a range of 0.3 to 30 mM).

Many diatoms contain a considerable amount of EPA (Table 10.3). Diatoms need silicate to form their frustules (cell walls composed of amorphous silica). Silicon metabolism in diatoms has been reviewed recently (72). The effect of silicate on the growth of diatoms usually follows the Monod equation (73). Under silicate-deficient conditions, the diatom uses its intracellular silicon pool to support its physiological activities (74). The lipid contents of several marine diatoms increase with decreased supply of silicate to the medium (75,76). Similarly, the EPA content of *N. laevis* increased when silicate became the limiting factor (73). The reason for this phenomenon is that in silicate-limited cultures, the cell tends to alter its metabolism and divert energy, that was previously allocated for silicate uptake, into lipid storage (77).

Environmental Factors. Photosynthesis requires light; efficient photosynthesis requires an abundance of light to all cells. The effects of light intensity and the light/dark cycle on microalgal growth and n-3 PUFA production have been extensively investigated (45,54,66,78,79). Vazhappilly and Chen (63,64) reported that EPA contents of many microalgae were lower in heterotrophic cultures than in photoautotrophic cultures; their results indicated that the biosynthesis of n-3 PUFA was enhanced under light but that faster growth was obtained under heterotrophic conditions (60). It appears that cultivation of mixtrophic cultures under a light/dark regime could yield optimal EPA productivities. This, however, requires further investigation.

Temperature is a very important factor affecting cell growth, lipid composition, and n-3 PUFA formation of microalgae. Low-temperature stress leads to a relatively high n-3 PUFA content because of the higher level of intracellular O_2, as the enzymes responsible for PUFA desaturation and elongation depend on the availability of O_2 (68,80).

A temperature shift strategy has been employed to enhance overall n-3 PUFA (including EPA) production because the optimal temperature for microalgal growth is often higher than that for n-3 PUFA formation. Such a phenomenon has been observed in many different algal species including *P. cruentum* (62), *Nannochloropsis* sp. (44), and *Pythium irregulare* (71). However, Ohta *et al.* (54) observed that the optimal temperature for the growth of *Porphyridium purpureum* also yields the highest EPA content. These results suggest that the effect of temperature on cell growth and EPA production should be studied for individual microalgal species.

pH is another physico-chemical factor affecting the production of n-3 PUFA by microalgae. Stinson *et al.* (71) studied the effect of initial pH on the EPA yield by *Pythium irregulare*. Although initial pH values were quite different, the pH values were similar at the end of cultivation. However, in cultures of *P. tricornutum*, the final pH values were quite different when the initial pH values ranged from 6.0 to 8.8. Although the cell dry wt concentrations were almost identical at different pH values, the EPA yield reached a maximum when the initial pH was 7.6 (51).

High levels of EPA have been detected in many species of marine microalgae. In contrast, very few species of freshwater algae contain high levels of EPA. Medium salinity may influence the physiological properties of marine microalgae. A green microalga *Dunaliella* sp. has been investigated in detail as to how medium salinity can affect its FA composition. The content of n-3 PUFA decreased as the medium salinity increased (81). In contrast, Seto *et al.* (45) investigated the effect of salt on the growth rate and FA composition of *Chlorella minutissima* by adding various amounts of seawater concentrate (SWC) or NaCl to an initially salt-free medium. Their results showed that cells grown in SWC- or NaCl-enriched medium contained higher percentages of EPA. In cultures of the marine diatom *N. laevis*, EPA yield was the highest at half the salinity of the artificial seawater (82).

Cultivation Systems for Microalgae

An efficient large-scale cultivation system is needed in order to explore a process to commercially produce EPA (83). Although most microalgal species are obligate photoautotrophs that require light for growth, a number of microalgae are capable of heterotrophic growth in the dark with one or more organic substrates as their sole carbon and energy source. For this type of microalgae, fermentation technology can be adopted and modified for large-scale production of microalgal products.

Photoautotrophic Cultivation Systems. Mass cultivation of microalgae originates from the development of open ponds, the oldest and simplest systems in which algae are cultured under conditions identical to the natural environment. Generally, commercial scale-up of the open ponds is difficult because of problems with contamination by unwanted algae, bacteria, and predatory protozoa (60). In addition, optimal

culture conditions are difficult to maintain in open ponds, and recovery of the biomass from dilute culture is expensive (10). As a result, only a few algal genera that grow in a selective and specialized environment have been successfully cultivated in open ponds. Cohen and Heimer (84) reported that EPA productivity of *P. cruentum* in open ponds was only 0.5 $mg \cdot L^{-1} \cdot d^{-1}$ in winter and 1 $mg \cdot L^{-1} \cdot d^{-1}$ in summer. It has been estimated that EPA productivity in open ponds can reach 4–8 $mg \cdot L^{-1} \cdot d^{-1}$ (37) at best.

Closed algal photobioreactors have been employed to overcome the problems encountered in open ponds (85,86). These systems are made of transparent plastics and generally placed outdoors for illumination by natural light, and the cultivation vessels have a large surface-to-volume ratio (Fig. 10.4). Although enclosed systems can reduce the chances of contamination, the growth of microalgae is still suboptimal due to variations in temperature and light intensity.

In principle, enclosed photobioreactors with artificial light and a separate CO_2 supply are similar to conventional fermentors. Some photobioreactors also have O_2 removal devices to reduce the toxic effect of high O_2 concentrations on algal growth (85,88). Photobioreactors have been employed to produce EPA from microalgae, such as *Nannochloropsis* sp. (89), *Monodus subterraneus* (43), and *P. tricornutum*

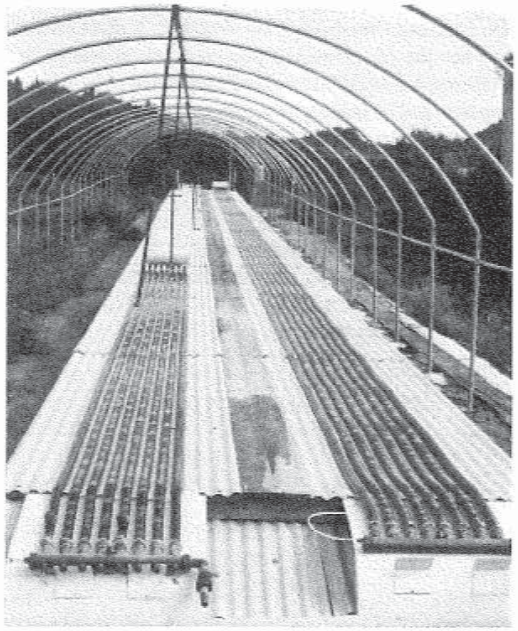


Fig. 10.4. A typical photobioreactor for outdoor cultivation of *Nannochloropsis* sp. for EPA production. Used with permission. *Source:* Reference 87.

(24,52,90). A high EPA productivity of almost 60 $mg \cdot L^{-1} \cdot d^{-1}$ was obtained in the culture of *M. subterraneus* (43). Enclosed photobioreactors, though, have several disadvantages, such as difficult scale-up, complex configuration and construction, and high capital cost. Moreover, light limitation cannot be entirely overcome, since light penetration into the culture is inversely proportional to the cell concentration (91). Possible film buildup on the plastic surface of the photobioreactors may further decrease light uptake by the algal cells.

Heterotrophic Cultivation Systems. In microalgal culture, heterotrophic growth can be a cost-effective alternative to photoautotrophic growth (60). In heterotrophic culture, organic carbon sources, such as sugars or organic acids, can be used as the sole carbon and energy source. This mode of culture eliminates the requirement for light and, therefore, offers the possibility of greatly increasing cell density and productivity in batch culture. A heterotrophic batch culture may be further modified to become a high cell density culture, such as fed-batch, chemostat, or perfusion culture. The development of high cell-density cultures for EPA production would also lead to a lower cost for EPA recovery and purification.

Many microalgae can grow rapidly heterotrophically (63,64). Generally, an organism used for heterotrophic production should possess the following characteristics: the ability to divide and metabolize in the dark; the ability to grow on inexpensive and easily sterilized media; the ability to adapt rapidly to a new environment (short or no lag phase when inoculated to fresh media); and the ability to withstand hydrodynamic stresses in fermenters and peripheral equipment. Some diatoms can also produce EPA heterotrophically (47,57); this indicates that culturing them in this way may provide an effective and feasible means for the large-scale production of EPA.

Cultivation Strategies for EPA Production by Microalgae

The competitiveness of microalgae-derived EPA over fish oil EPA depends largely on the high EPA yield and productivity attained by microalgal cultures. The commonly used cultivation mode is batch culture. To achieve high cell density of microalgae and, thus, high yield and productivity of EPA, strategies such as fed-batch, continuous and perfusion cultures may be employed.

Fed-Batch Culture. Fed-batch is a commonly used culture technique in the fermentation industry. This culturing mode can attain a high cell density by avoiding the limitation of, or inhibition by, substrates. A fed-batch process has been developed for high cell-density culture of *N. laevis* for enhanced production of EPA (92). The fed-batch culture led to a cell dry wt concentration of 22.1 $g \cdot L^{-1}$ and an EPA yield of 695 $mg \cdot L^{-1}$, both of which are much higher than those achieved in batch cultures (92).

Although a fed-batch culture can eliminate substrate limitations, it cannot overcome the inhibition caused by toxic metabolites produced by the cells. When the cell density reaches a high level, the accumulation of toxic metabolites becomes signifi-

cant and, as a result, limits further growth of the cells. Therefore, other efficient culture techniques should be developed in order to enhance EPA yield and productivity.

Continuous Culture. Continuous culture allows a higher EPA productivity than batch or fed-batch culture. It is also used as an important tool to study the basic physiological behavior of algal cells since kinetic parameters, such as specific growth rate, cell density, and productivity, can be kept constant at steady state. Continuous culture of *Isochrysis galbana* was previously investigated to achieve high EPA productivity (93–95). Similarly, continuous cultures of *P. tricornutum* and *N. laevis* have been reported to produce EPA (24,58,90,96).

A large number of extracellular metabolic products have been identified in microalgae in general (97,98) and in diatoms in particular (99). To avoid growth inhibition from toxic by-products and the limitation of substrate, a continuous culture with cell-recycle, (called perfusion culture) has been developed in heterotrophic cultures of *N. laevis* (100). The perfusion culture is further modified to permit cell bleeding during the perfusion operation (Fig. 10.5). This strategy allows continuous harvesting of the algal cells while simultaneously removing inhibitory compounds during cultivation. Thus, high EPA productivity can be achieved by continuously harvesting the EPA-containing algal biomass (101). The perfusion-cell bleeding culture allows a much higher EPA productivity than the simple perfusion culture does. At a bleeding rate of 0.67 d^{-1} and a perfusion rate of 0.6 d^{-1}, the EPA productivity achieved is 175 $mg \cdot L^{-1} \cdot d^{-1}$ (Table 10.4). This EPA productivity is the highest ever reported in microalgal cultures.

Prospects of EPA Production by Microorganisms

In the past decade, more and more clinical research has shown the beneficial effects of EPA and DHA on the treatment of various human cardiovascular and immune system

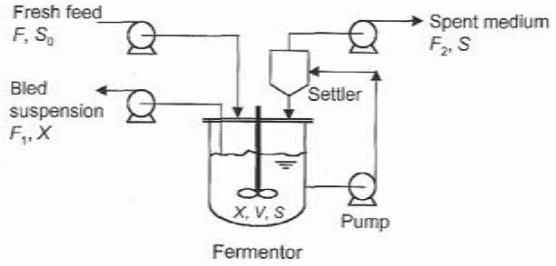


Fig. 10.5. Schematic diagram of the perfusion culture with cell bleeding system. X, cell concentration; V, culture volume; S, glucose concentration in medium; S_0, glucose concentration in feed; F, flow rate of feed; F_1, flow rate of bleeding; F_2, flow rate of perfusion. *Source:* Reference 111, with permission from Elsevier.

diseases. EPA and DHA products have now been well recognized by consumers. To date, microalgae-derived DHA products have already been introduced into the infant formula market (see Chapter 6). In contrast, fish oil is still the sole commercial source of EPA. The main reason impeding the commercialization of microbial EPA production is that EPA yield and productivity are still very low; this results in high operational and recovery/purification costs. For example, the current DHA production process using the dinoflagellate, *Crypthecodinium cohnii*, has achieved a productivity of around 50 $mg \cdot L^{-1} \cdot h^{-1}$ by simple fed-batch cultivation (see Chapters 6 and 13). In contrast, the highest EPA productivity is only 7.25 $mg \cdot L^{-1} \cdot h^{-1}$ using a relatively complicated perfusion-cell bleeding culture technique. A comparison of DHA and EPA production from microalgae is given in Table 10.5. The low EPA productivity is attributed to both the low cellular EPA content and the relatively low growth rate of algae (Table 10.5). Thus, the microalgae-derived EPA product is not yet commercially feasible. Another reason for microbial EPA production being rarely commercialized is that EPA-producing algae have relatively complex FA profiles compared with DHA-containing algae (82,105). Low EPA productivity with relative complex FA composition means high costs for the operation and for EPA recovery; this limits the application of microalgae to EPA production.

Several aspects of research need to be performed toward the commercialization of EPA production by microalgae. First, research should be focused on heterotrophic growth of microalgae. Under photoautotrophic conditions, the growth rate of microalgae is very low, and light limitation further limits cell growth. EPA productivity could

TABLE 10.4
Comparison of EPA Productivity of Microalgae Under Various Culture Conditions[a]

Organisms	Culture Vessels	Culture Modes	EPA productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	Reference
P. tricornutum	Glass tubes	Batch	19.0	51
P. tricornutum	Glass tanks	Continuous	25.1	102
Isochrysis galbana	Fermentors	Continuous	7.2	93
I. galbana	Fermentors	Continuous	15.3	94
M. subterraneus	Erlenmeyer flasks	Continuous	25.7	78
M. subterraneus	Flat plate reactors	Semi-continuous	58.9	43
I. galbana	Glass tubes	Semi-continuous	4.6	95
P. tricornutum	Glass tubes	Semi-continuous	5.2	103
Porphyridium cruentum	Flasks	Batch	3.6	79
I. galbana	Cylindrical fermentors	Continuous	23.8	104
Nannochloropsis sp.	Tubular photobioreactors	Continuous	32.0	87
P. tricornutum[b]	Glass vessels	Batch	33.5	56
Nitzschia laevis[c]	Fermentors	Perfusion-cell bleeding	174.6	101

[a]Unless specified, the microalgae were grown photoautotrophically.
[b]The microalga was grown in mixotrophic growth conditions, i.e., grown under light with glycerol as carbon source.
[c]The microalga was grown heterotrophically.
For abbreviations see Table 10.1.
Source: Reference 111, with permission from Elsevier.

TABLE 10.5
Comparison of EPA and DHA Production by Microalgae

Alga	Product	Culture Mode[a]	Substrate	Biomass ($g \cdot L^{-1}$)	EPA/DHA Content (% dry wt)	EPA/DHA Yield ($g \cdot L^{-1}$)	EPA/DHA Productivity ($mg \cdot L^{-1} \cdot h^{-1}$)	References
Cryptheconlinum cohnii	DHA	Fed-batch	Glucose	26	6.9	1.7	14	105
C. cohnii	DHA	Fed-batch	Acetate	61	15.7	9.5	45	105
C. cohnii	DHA	Fed-batch	Ethanol	83	14.1	11.7	53	106
N. laevis	EPA	Fed-batch	Glucose	40	3.0	1.1	3.1	92
N. laevis	EPA	Perfusion bleeding	Glucose	10	2.6	0.3	7.3	101

[a]Heterotrophic culture mode was employed for all cultures.
For abbreviations see Tables 10.1 and 10.2.

be significantly enhanced if the selected algae could grow heterotrophically. In addition, high cell-density culture techniques may be applied using industrial-scale fermentors. Although fermentors used in heterotrophic culture are more expensive than photobioreactors, the enhancement of EPA productivity could pay for the high capital cost of fermentors.

Second, microalgal strains can be improved by mutation and genetic engineering. For example, Cohen *et al.* (107) selected cell lines of *Spirulina platensis* and *P. cruentum* with the herbicide Sandoz 9785 (see Chapter 4). The herbicide-resistant mutant of *P. cruentum* was able to over produce EPA. Lopez-Alonso *et al.* (108) also selected mutant strains of *P. tricornutum* for EPA production, and one mutant (II242) contained 44% more EPA than the wild type.

The first cloning and expression of the large heterologous EPA synthesis gene cluster was accomplished in a marine cyanobacterium; *Synechococcus* sp. (109). The EPA synthesis gene cluster (approximately 38 kb, GenBank accession number U73935) was isolated from a marine bacterium, *Shewanella putrefaciens* (11). It contained eight open reading frames, three of which include the genes encoding carbon chain elongation enzymes, while the rest include desaturase genes (11). Yu *et al.* (110) further reported that another transconjugant *Synechococcus* sp. could produce a maximum EPA yield of 3.9 $mg \cdot L^{-1}$. Although the EPA yields obtained in these studies remained low (109,110), they demonstrated the possibility of employing this approach to modify lipid composition; this might lead to further improvement in EPA productivity. Some remarkable findings in making transgenic microalgae for enhanced EPA production have been recently reported (65). These researchers introduced the gene encoding a glucose transporter (glut1 or hup1) into an obligate photoautotrophic diatom *P. tricornutum* to enable the alga to thrive on exogenous glucose and produce EPA in the absence of light.

Conclusion

EPA is a precursor of a large variety of bioactive metabolites and performs diverse physiological functions in the human body. Evidence of the beneficial effects of EPA has brought this FA to the attention of food and pharmaceutical markets worldwide. The increasing applications for EPA and its inadequate conventional sources (i.e., fish oils) have led to an extensive search for alternative sources including microalgae, lower fungi, and marine bacteria. Among the microorganisms known to produce EPA, microalgae are the richest source.

The EPA production potential of microalgae depends on the characteristics of the specific algal species and the cultivation strategies developed. Heterotrophic cultivation is a cost-effective means to produce EPA on a large scale. However, investigations of heterotrophic EPA production from microalgae are still in their infancy. Therefore, an in-depth understanding of the factors that affect EPA production is needed. In the future, application of genetically modified microorganisms may be the most efficient means of attaining improved production of EPA.

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Chapter 11

Safety Evaluation of Single Cell Oils and the Regulatory Requirements for Use as Food Ingredients

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Introduction

Safety refers to a "reasonable certainty of no harm" according to Title 21 of the United States Code (Section §348). Safety is an intellectual concept and not an inherent property of a substance. Safety assessment is a continual ongoing process, not an absolute determination based on a single point in time. The relationship between biology and safety is mediated through concepts of harm, benefit, and risk. Risk analysis forms the basis on which food safety policy is based. The three components of risk analysis involve risk assessment (scientific advice and information analysis), risk management (regulation and control), and risk communication. As such, the concepts of safety, safety assessment, and risk analysis are influenced by many different individuals, organizations, and regulatory authorities, and encompass several intellectual disciplines.

In the U.S., the Food and Drug Administration (FDA) has the primary responsibility for regulating new food ingredients. Manufacturers may propose the addition of new food ingredients in the U.S. by either filing a food additive petition with the FDA to request a formal premarket review, or making a generally recognized as safe (GRAS) determination. It is important to note that it is the use of a substance, rather than the substance itself, that is eligible for the GRAS determination. The European Union (EU) has regulations in place for food additives, novel foods, and genetically modified organisms. Manufacturers wishing to obtain approval for use of a new ingredient in the EU traditionally approach the competent authority of a Member State to consult on the appropriate (e.g., novel food application) approach to obtain premarket clearance. The Novel Foods Regulation provides two routes to authorize novel foods, a full procedure and a simplified procedure based on the concept of "substantial equivalence."

The safety of single cell oils (SCO) has been evaluated and discussed over the past decades in numerous forums including published articles, presentations at scientific conferences, by expert panels qualified by training and experience to evaluate the safety of food ingredients, and by regulatory bodies around the world. A large number of nonclinical and clinical studies have been published on DHASCO® [docosahexaenoic acid (DHA) oil derived from *Crypthecodinium cohnii*] and ARASCO®

[arachidonic acid (AA) oil derived from *Mortierella alpina*] supporting safe use of these oils in infant formulas. In addition, these oils have been successfully commercialized for use in infant formulas around the world. More recently, published studies documenting the nonclinical safety evaluation of DHA oil derived from *Schizochytrium* sp. (DHASCO-S) and *Ulkenia* sp. (DHA45 oil) have been used to support safe use of these oils in a number of food applications.

The intent of this chapter is to review elements that comprise an overall safety evaluation of a SCO intended for use as a food ingredient, referencing published information as available. A discussion is also presented on the regulatory pathway to obtain premarket clearance of SCO for use as food ingredients in the U.S. and in the EU citing some of the more recent published approvals for these oils.

Safety Evaluation

Safety evaluation of food ingredients, including SCO, is based on the "reasonable certainty of no harm." Sections 201(s), 201(z), 409, and 412 of the Federal Food, Drug, and Cosmetic Act (FDCA) and the U.S. FDA's *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*, also known as the Redbook, (1,2) are the regulations and guidelines that are used to assess the safety of food ingredients. The FDA Redbook was prepared to assist in the design of protocols for animal studies conducted to test the safety of food ingredients and includes detailed guidelines to test the effects of food ingredients.

In the U.S., there are two routes to obtaining regulatory clearance for a food ingredient. A food additive petition process requires premarket review and approval by FDA, whereas under the GRAS notification proposed rule a manufacturer can determine that a substance is GRAS if there is scientific consensus among qualified experts about its safety under the intended conditions of use. The manufacturer may then notify FDA, and a letter of no objection is issued if the agency has no questions. The main difference between a food additive petition and a GRAS notification is that a food additive petition places the responsibility of declaring that a substance is safe and approved under the conditions of use with the regulatory agency; the GRAS notification process places the responsibility of demonstrating that a substance is GRAS, and therefore safe under the conditions of use, with the manufacturer. The GRAS notification and the food additive petition procedures are intended to ensure the safety (a reasonable certainty of no harm), not the efficacy, of the proposed ingredient. The majority of SCO, utilized as new food ingredients, are likely to follow the GRAS process to establish safety.

The EC Scientific Committee on Foods [now the European Food Safety Authority (EFSA)] has prepared a guidance document with a decision tree process to help determine what type of safety data should be included in a novel food submission. Data may typically range from a full food additive type toxicology package to merely analytical comparisons. A selected list of laws and regulations pertaining to new food ingredients in the U.S. and the EU are given in Table 11.1.

TABLE 11.1
Selected Laws, Regulations, and Recommendations Related to New Food Ingredients

Country	Regulation	Applicability
United States	FDCA (21 USC §301)	Food additive, GRAS
	Substances Generally Recognized as Safe (GRAS) (21 CFR §170, 184, 186, and 570)	GRAS
	Requirement for premarket notification; (62 Fed. Reg. 49886-49892; 21 CFR §190.6)	Dietary supplement
European Union	Council Directive 89/107/EEC (OJ L 40/27, 2.11.1989)	Food additive
	European Parliament and Council Directive 94/34/EC (OJ L 237/1, 9.10.1994)	Food additive
	Regulation (EC) No. 258/97 of the European Parliament and of the Council (OJ L 43/1, 2.14.1997)	Novel foods, novel food ingredients
	Commission Regulation (EC) No. 1852/2001 (OJ L 253/17, 9.21.2001)	Confidential information for novel foods submission
	Commission Recommendation No. 97/618/EC (OJ L 253/1, 9.16.1997)	Scientific aspects and information for novel foods
	Regulation (EC) No. 178/2002 of the European Parliament and of the Council (OJ L 31/1, 2.1.2002)	General principles and requirements of food law, establishment of the European Food Safety Authority

CFR, code of federal regulations; FDCA, Food, Drug, and Cosmetic Act; USC, United States code; Fed. Reg., federal regulation; OJ, Official Journal of European Communities.

There are many elements that go into the overall evaluation of safety of a SCO. Under current regulations, no measures of efficacy (i.e., health benefits) for SCO in question are required. Section 409 of the FDCA deals with safety of food additives independent of efficacy, because efficacy has traditionally been considered a property of drugs and inappropriate for foods unless accompanied by an authorized health claim. While safety and efficacy are clearly separated under U.S. regulations, they may not be so clearly delineated elsewhere. This section will focus on the safety of SCO with acknowledgment that safety and efficacy are not necessarily a mutually exclusive attribute.

Safety Assessment Approach

SCO are unique due to the non-traditional nature of the source organism used for their production and perhaps certain compositional attributes; therefore, a flexible approach to determine overall safe use in a given application is important. However, there are a few common technical elements to consider. Chemical and physical characterization of the product is important since safety considerations often revolve around what is known

regarding the product and its individual components. In addition to characterization of major constituents, minor components of complex products derived from SCO must be examined to determine their potential for toxicity, including the possibility for naturally occurring toxins (from the source organism), heavy metals, hazardous levels of pathogenic microorganisms, as well as potential by-products formed from degradation pathways or introduced from production processing. Knowledge of chemical composition/structure along with intended uses and levels of exposure are important to determine an appropriate level of toxicological assessment. FDA has prepared guidance to recommend the minimum toxicity tests to be performed for safety evaluation of food additives based on levels of concern. Concern levels, as determined by the agency, are "relative measures of the degree to which the use of an additive may present a hazard to human health" (2). The concern level is based on the extent of human exposure (dose) and the toxicological effects on biological systems. There are three broad bands of concern levels, with concern level three representing the highest probable risk to human health, concern level one the lowest probable risk, and concern level two intermediate between the two. The concern levels are used as a starting point to recommend toxicity tests for a particular substance at a given dose or exposure level and are listed in Table 11.2.

Safety of Source Organisms

The use of SCO for human consumption including their use in infant formulas (both preterm and term) has a relatively short history, but SCO may be considered similar to

TABLE 11.2
Summary of Toxicity Tests Recommended for Different Levels of Concern for Direct Food Additives

	Concern Levels		
Toxicity Tests	I	II	III
Acute Oral Study—Rodent	—	X[a]	X[a]
Short-Term Feeding Study—Rodent	X	—	—
Subchronic Feeding Study—Rodent with *in utero* Exposure	—	—	—
Subchronic Feeding Study—Rodent	—	X	X[a]
Subchronic Feeding Study—Nonrodent	—	X	—
Lifetime Feeding Study—Rodent with in utero Exposure for Carcinogenesis and Chronic Toxicity	—	—	X
Lifetime Feeding Study—Rodent for Carcinogenesis	—	—	X
Short-Term Feeding Study—Nonrodent	—	—	X
Multigenerational Reproduction Feeding Study with Teratology Phase—Rodent	—	X	X
Teratology Study	—	X[b]	X[b]
Short-Term Tests for Carcinogenic Potential	X	X	X
Metabolism Studies	—	—	X[c]

[a]If needed as preliminary to further study.
[b]If indicated by available data or information.
[c]Suggested.
Source: Reference 3.

various plant oils with regard to certain compositional attributes. The main difference between SCO and plant oils and the source of their "novelty" in certain regulatory jurisdictions is concerned with the source organism used for their production. The following paragraphs describe some of the commercially available SCO along with a description of their source organism, with reference to published articles supporting their safety.

DHASCO is the trade name used by Martek Biosciences Corporation (Martek) to describe the DHA SCO produced by the microalga, *C. cohnii* (see Chapter 6). This single cell heterotrophic organism is a marine algal species that has been extensively studied in both laboratory and commercial environments. *C. cohnii* is a member of the Dinophyta (dinoflagellates), a distinct phylum of unicellular eukaryotic microalgae consisting of approximately 2,000 species (4). *C. cohnii* is non-pathogenic in man and animals, and the organism does not produce any toxins. There are a small number of photosynthetic species of Dinophyta that are known to produce a group of closely related toxins (5) that are passed through the food chain *via* zooplankton and can contaminate fish and bivalves. However, these toxin-producing species are few in number, and there are no known heterotrophic species of the Dinophyta that are toxin producers. In the many reports of *C. cohnii* in culture over the last 30 years, there has never been any indication that *C. cohnii* produces any toxin, nor is it related to any toxin-producing species (6).

ARASCO is the tradename used by Martek for the AA SCO produced through a fermentation process using the common soil fungus *M. alpina* (see Chapter 5). *M. alpina* has been studied at length and is not pathogenic to humans nor does it produce mycotoxins harmful to humans or animals (7). *Mortierella* species have been well studied for many years in both laboratory and commercial environments and their morphology, biochemistry, and physiology is well documented. *M. alpina* has also been described in Japanese publications and patents as a potential source of AA and, as a consequence, it has been the subject of recent laboratory investigation. In none of the recent work has there ever been any report of pathogenicity or toxigenicity to humans or animals by *M. alpina* (8,9); this is consistent with earlier studies.

DHASCO-S is Martek's tradename for DHA oil derived from the heterotrophic fermentation of the marine alga, *Schizochytrium* sp. (see Chapter 3). *Schizochytrium* sp. is a thraustochytrid and a member of the Chromista kingdom (Stramenopila) that includes the golden algae, diatoms, yellow-green algae, haptophyte and cryptophyte algae, and oomycetes. There are no reports of this organism producing toxic chemicals nor is it pathogenic. The two toxic compounds known to be produced in the Chromista (to which *Schizochytrium* sp. belongs) are largely restricted to two genera (domoic acid in *Pseudonitzschia* and prymnesin in *Prymnesium* sp.) that are in a separate class and phylum, respectively, from the thraustochytrids. No evidence of the two toxic compounds produced in the Chromista was found in *Schizochytrium* sp. algae using chemical and biological assays. Chemical and biological analysis of the production strain confirmed the absence of common algal toxins (10).

DHA45 oil is described by Kroes *et al.* (11) as a refined oil produced through a fermentation process using a strain of the marine protist, *Ulkenia* sp. This organism is

stated to be a member of the non-pathogenic and non-toxigenic family of Thraustochytriaceae (11).

Safety of the SCO Components

Evaluation of the overall safety of a SCO involves the review of the safety of the identified components of the oil. SCO are typically comprised of fatty acids (FA) found esterified to glycerol (e.g., triacyl)glycerols also referred to as triglycerides) and may contain minor amounts of other lipid classes (e.g., steryl esters, free sterols, and carotenoids). Identified FA present in commercialized SCO have been described as components of normal human diets or FA metabolites. Sterols identified in SCO are commonly found in several traditional food sources including animal fat, vegetable oil, and human milk or are part of the normal metabolic pathway of cholesterol biosynthesis in man. A safe history of use of the individual FA and sterols is further supported as a result of their abundant natural presence in food and the small quantities expected to be consumed, the extensive knowledge of the absorption, distribution, metabolism, and excretion in mammalian species and published safety information on the specific FA, sterols, and similar compounds.

Nonclinical Toxicology of SCO

Several manufacturers of SCO, as well as finished product formulators (e.g., infant formula companies) and others, have performed toxicology studies on DHASCO, ARASCO, and other AA-containing oils derived from *M. alpina* to support the safe use of the oils in infant formula and other applications at specified use levels. As a result, there is redundancy in the standard toxicological testing, allowing for an in-depth and broad assessment of the oils. Following is a review of some of the nonclinical studies performed on SCO with specific reference to published articles. There have been a number of reports involving dietary supplementation of various mammalian species, including mice, rats, pigs, cats, dogs, monkeys, and baboons, with SCO; however, specific references to these published studies are beyond the scope of this review as is a review of the numerous human clinical studies supporting the efficacy of these oils.

Studies conducted using DHASCO include *in vitro* mutagenicity (12) and genotoxicity studies (12), and a variety of animal studies (rodent and nonrodent), such as acute (13), short-term, subchronic (13–19), developmental, and reproductive studies (20). These studies have been recently reviewed (21), and an overview of some of the published studies with toxicological outcome (used in part to support safe use of DHASCO) is presented in Table 11.3.

The studies conducted using ARASCO and other AA-containing oils from *M. alpina* include *in vitro* mutagenicity (12) and genotoxicity studies (12), and a variety of animal studies (rodent and nonrodent), such as acute (13), short-term, subchronic (13,14,16–19,22–25), developmental and reproductive studies (20). An overview of these studies supporting safe use of ARASCO and AA-containing oils from *M. alpina* is presented in Table 11.3.

TABLE 11.3
Summary of Nonclinical Safety Studies Using DHASCO (Docosahexaenoic Acid [DHA] Oil Derived from *Crypthecodinium cohnii*), ARASCO [Arachidonic Acid (AA) Oil Derived from *Mortierella alpina*], and ARA Oils from *M. alpina*

Study	Max DHASCO Dose	Max[m] ARASCO Dose	Results	Reference
In vitro genetic toxicity				12
Ames mutagenicity	5000 μg/plate	5000 mg/plate	Neither oil was mutagenic	
Forward mutation	5000 μg/mL	4990 mg/mL	Neither oil was mutagenic	
Chromosomal aberration	5000 μg/mL	5010 mg/mL	Neither oil was clastogenic	
Acute toxicity				
Rat	20 g/kg bw/d	20 g/kg bw·d[a]	No deaths with either oil; LD_{50}[b] > 20 g/kg bw	13
Short-Term and Subchronic Toxicity				
Rat	1.25 g/kg bw·d[a]	2.5 g/kg bw·d[a]	Neither oil was toxic; NOAEL[c] = 1.25 g DHASCO and 2.5 g ARASCO per kg bw/d	14
Rat	3.2 g/kg bw·d[a]	6.3 g/kg bw·d[a]	Blend was not toxic; NOAEL[c] = 9 g of the blend/kg bw·d	14
Rat	1.25 g/kg bw·d[a]	2.5 g/kg bw·d[a]	Neither oil was toxic; NOAEL = 1.25 g DHASCO and 2.5 g ARASCO per kg bw/d	15,22
Rat	3 g/kg bw·d[a]	6 g/kg bw·d[a]	Blend was not toxic; NOAEL[c] = 9 g of the blend/kg bw·d	16
Piglet	0.1% w/w of total fat in diet		Growth not compromised; no alteration of liver fatty acid composition	17
Piglet	85 mg/100 kcal formula/d	175 mg/100 kcal formula/d	Not toxic; dose-related increases in DHA in target tissues but no other significant effect	18
Piglet	0.3 g DHA/100 g total fatty acids	0.6 g ARA/ 100 g total fatty acids	No difference in liver histology and liver enzymes compared to controls	19
Piglet		96 mg/100. kcal formula/d	No test article related effects	22
Rat		3 g/kg bw·d[a]	Not toxic; NOAEL[c] = 3 g AA oil per kg bw/d	24
Rat		4.9 g/kg bw·d[a]	NOEL[d] = 1 g/kg bw·d (mid-dose level)	25
Developmental Toxicity				
Rat	1.25 g/kg bw·d[a]	2.5 g/kg bw·d[a]	Not teratogenic; NOAEL[c] = 1.25 g DHASCO and 2.5 g ARASCO per kg bw/d	20

[a]kg/bw·d = kilograms per body weight per day
[b]LD_{50} = mean lethal dose—the dose at which 50% of the animals die.
[c]NOAEL, no observable adverse effect level (the highest dose at which no adverse effects are experienced).
[d]NOEL, no observable effect level (the highest dose at which no effects are experienced).

A recent series of articles describing the toxicological assessment of *Schizochtyrium* sp. and DHA oil derived from *Schizochytrium* sp. have been published. These studies describe results of subchronic feeding to rodents (26) and nonrodents (27), developmental toxicity evaluation in rodent and nonrodent species (28), a reproduction study (29), and *in vitro* mutagenicity and genotoxicity studies (30).

A review of the safety of DHA45-oil derived from the marine protist, *Ulkenia* sp., has been published (11). This study describes results of genotoxicity and acute, subchronic, and reproductive studies of the oil and the marine protist.

These toxicological studies all support the safe use of SCO. Although expected findings associated with a high-fat diet or with polyunsaturated FA (PUFA)-containing oils in general were detected in some of the rodent studies, no unexpected treatment-related, dose-dependent, adverse toxicological effects were attributed specifically to the SCO tested. The "no observable adverse effect levels" were generally the highest dose administered, with the maximum doses generally limited by the method of administration. More recently, the neonatal piglet has been used as a surrogate model for human neonates to assess possible adverse effects of specific sources of SCO. Results from several of these studies (see Table 11.3), used in part as a second level assessment of specific organ systems, have been published (18,19,22). The findings from these neonatal piglet studies did not reveal any clinical chemistry, hematology, organ weight, or histopathologic indications of toxic effects; all support the safety of SCO sources of DHA and AA for use in infant formula.

SCO Clinical Studies

DHASCO and ARASCO have been the subject of over 10 well-controlled infant clinical intervention trials (31–43). None of the infant studies, including those conducted with vulnerable preterm infants, have reported adverse effects of DHASCO or ARASCO when added to infant formulas at levels similar to those found in breast milk. Safety outcomes were very carefully monitored in several of the larger studies (34–36,40) conducted for regulatory purposes by infant-formula manufacturers to assess the safety of new formulas containing DHASCO and ARASCO. There were no adverse effects on hematology or tolerance and no differences in adverse events between treatment and control infants in these studies. Taken together, these studies demonstrate the safe use of DHASCO and ARASCO when consumed by preterm and term infants.

High-dose clinical studies performed with DHASCO and ARASCO that included safety outcomes in normal healthy adults have been reported (44–60) along with studies performed in pregnant or lactating women (61–66). No serious treatment-related adverse effects have been attributed to the use of DHASCO or ARASCO oil in normal healthy adults, including pregnant and lactating women. Mild gastrointestinal symptoms, including eructation, have been reported with DHASCO oil in some studies.

Postmarket Surveillance

It is the FDA's view that the evaluation for safe use of a food ingredient is a time-dependent judgment based on general scientific knowledge as well as specific data and information about the ingredient. Based, in part, on this view and an ongoing obligation to continually monitor the safety of GRAS ingredients, SCO manufacturers may implement a postmarket surveillance program. Likewise, infant-formula manufacturers that utilize DHASCO and ARASCO in infant formula may conduct their own postmarket surveillance. One component of a postmarket surveillance program involves assessment of reports made through toll-free numbers or *via* the Internet. This is often referred to as passive surveillance. More active surveillance programs may take the form of follow-on clinical studies in populations and sub-populations of interest (e.g., infants, children, and pregnant or lactating women). In the case of Martek, a company with an active clinical research program, clinical studies are routinely conducted with a safety component in mind. Expected adverse effects, unexpected adverse effects, and serious adverse effects attributed to the product are monitored, reported, and collected in databases that allow for ongoing, continual tracking of product safety profiles. These data are reviewed by physicians and experts qualified by scientific training and experience to determine the consequences of exposure and forms part of the postmarket safety assessment program.

Regulatory Requirements

United States

Food Ingredients. In response to public concern about the increased use of chemicals in foods and food processing, Congress passed the 1958 Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act (FDCA). The basic purpose of the amendment was to require that an additive manufacturer demonstrate the safety of the additive to the FDA before the new additive could be used in food. The amendment defined the terms "food additive" [FDCA §201(s)] and "unsafe food additive" [FDCA §409(a)], and established a premarket approval process for food additives [FDCA §409(b) through (h)].

When passing the amendment, the U.S. Congress recognized that many substances intentionally added to food would not require a formal premarket review by the FDA to ensure their safety. For example, the safety of some substances could be established by a long history of use in food or by virtue of the nature of the substances, their customary or projected conditions of use, and the information generally available to scientists. Therefore, Congress enacted a two-step definition of "food additive" [FDCA §201(s)]. The first step broadly includes any substance, the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of food. The second step, however, excludes from the definition of "food additive" substances that are generally recognized as having been adequately shown through scientific procedures (or in the case of substances used in food prior to January 1, 1958, through either scientific procedures or through experience based on common use in food) to be safe under the conditions of their intended use by experts qualified by scientific training and experience to evaluate their safety. This exception to the food additive definition

came to be known as the GRAS exemption. Many substances that are commonly used in foods today (e.g., vegetable oil) are legally marketed under the GRAS exemption.

One of the key elements of the GRAS exemption is that a substance that is GRAS for a particular use may be lawfully marketed for that use without FDA review or approval. Nevertheless, many manufacturers have found it useful to have a statement from the FDA agreeing with the manufacturer's GRAS determination. Initially, the FDA issued informal "opinion letters" concerning the GRAS status of substances. The opinion letters, however, were issued only to the specific person requesting the letter and therefore did not provide industry-wide notification of the agency's GRAS decision. To address this and other concerns, the FDA adopted the GRAS affirmation petition process. This was a voluntary administrative process whereby manufacturers could petition the FDA to affirm that a substance was GRAS under certain conditions of use. If the FDA agreed with the petitioner's GRAS determination, a regulation was published in the Code of Federal Regulations affirming the GRAS status of the substance. The GRAS affirmation petition process was intended to provide a mechanism for official recognition of lawfully made GRAS determinations. To the extent that a person elected to submit a GRAS affirmation petition, the process facilitated awareness by the FDA as well as the domestic and international food industry, of lawful independent GRAS determinations.

The GRAS affirmation petition process, however, turned out to be extremely resource-intensive, involving a comprehensive review of each petition and requiring it to undergo a rule-making process for each substance affirmed as GRAS. As a result, GRAS petitions languished at the agency for years, even decades, without the publication of a final regulation. As a result of the problems encountered with the GRAS petition process, the FDA proposed a "GRAS notification" procedure in April, 1997 (see Table 11.1). This procedure was intended to replace the GRAS affirmation petition process. Under the GRAS notification procedure, FDA evaluates whether a GRAS "notice" provided by the manufacturer provides a sufficient basis for a GRAS determination and whether information in the notice or otherwise available to FDA raises issues that might lead the agency to question whether use of the substance is GRAS. Within 90 days of receipt of the notice, the FDA responds in writing as to whether it has identified a problem with the notice. To provide the industry with information on prior GRAS notices, the FDA publishes a list of all submitted GRAS notices, along with the agency's response, on the FDA web site.

Although the GRAS notification regulation has never been finalized, the FDA has adopted the procedure to replace the GRAS affirmation petition process. At the time of this writing, and since the publication of the GRAS notification proposed rule, the agency has received 146 GRAS notices (67) including several related to SCO (*vide infra*).

If a potential new ingredient cannot be determined to be GRAS, a manufacturer must file a petition proposing the issuance of a regulation prescribing the conditions under which the proposed additive may be safely used. The manufacturer supplies the FDA with all pertinent data, especially safety data, and the agency then conducts a

comprehensive review of all the safety data and determines if the ingredient is safe for its intended use.

Content of a GRAS Notification. The GRAS process is considered to be rigorous, flexible, credible, and transparent. To date, SCO manufacturers have followed a GRAS process to establish safety, since SCO possess a variety of characteristics amenable to this process. SCO are usually derived from novel sources or processes; such diversity requires safety considerations that are clear but not overly prescriptive because of the multitude of issues with each type of oil and organism from which it is derived.

Any person may notify the FDA of a claim that a particular use of a SCO is exempt from the statutory premarket approval requirements based on the notifier's determination that such use is GRAS. Within 30 days of receipt of a notice, the FDA will acknowledge receipt of the notice by informing the notifier in writing, and within 90 days of receipt of the notice, the FDA will respond to the notifier in writing. Copies of the GRAS exemption claim submitted to the agency along with a letter issued to the notifier acknowledging receipt of the notification and subsequent letter(s) issued by the agency regarding the notification are accessible for public review.

The FDA has provided guidance on how to submit a GRAS notification. The content of the notification shall include the following information: a claim, dated and signed by the notifier that a particular use of a substance is exempt from the premarket approval requirements of the FDCA because the notifier has determined that such substance is GRAS; detailed information about the identity of the substance, including methods of manufacturing (excluding any trade secrets and including substances of natural biological origin, source information such as genus and species), characteristic properties, any content of potential human toxicants, and specifications for food-grade materials; information on any self-limiting levels of use; and a detailed summary of the basis for the notifier's determination that a particular use of the notified substance is exempt from the premarket approval requirements of the FDCA because such use is GRAS. Such a determination may be based either on scientific procedures or on common use in food. For a GRAS determination based on scientific procedures, such a summary shall include a comprehensive discussion of and citations to generally available and accepted scientific data, information, methods, or principles that the notifier relies upon to establish safety; a comprehensive discussion of any investigation reports or other information that may appear to be inconsistent with the GRAS determination; and the basis for concluding that there is consensus among experts that there is reasonable certainty that the substance is not harmful under the intended conditions of use.

GRAS Status of SCO. Several GRAS notifications for SCO have been posted on the FDA web site. At the time of writing, three have been successfully reviewed by the agency (67).

In 2001, the FDA responded to Martek GRAS Notification GRN 000041 that DHASCO as a source of DHA derived from the microalgal species *C. cohnii* and

ARASCO as a source of AA derived from the soil fungus *M. alpina* are GRAS when added to term infant formulas. Based on the information provided by Martek, as well as other information available to the FDA, the agency had no questions regarding the conclusion that ARASCO and DHASCO are GRAS sources of AA and DHA under the intended conditions of use—i.e., when added to infant formulas for consumption by healthy term infants at a level of up to 1.25% of total dietary fat each and at a ratio of DHA to AA of 1:1 to 1:2 (68). In response to GRAS notice no. GRN 000080 (69), and based on information provided by Mead Johnson Nutritionals (one of the leading U.S. infant formula manufacturers) as well as other information available, the FDA had no questions regarding the conclusion that ARASCO is GRAS under the intended conditions of use—i.e., when used in combination with DHASCO and to use ARASCO at a 50% increase relative to that proposed by Martek in GRN 000041.

In the case of a new infant formula that contains a SCO (*e.g.*, DHASCO or ARASCO), the manufacturer of the infant formula must make a submission to the FDA, providing required assurances about the formula, at least 90 days before the formula is marketed under section 412 of the FDCA. An infant-formula manufacturer that intends to market an infant formula containing a new ingredient bears the responsibility for submission required by section 412, not the manufacturer of the ingredient itself.

GRAS notice no. GRN 000137 (10) was based on scientific procedures regarding the use of DHASCO-S as a direct food ingredient in specified food categories at specified use levels. Based on the information provided by Martek as well as other information available to the FDA, the agency had no questions regarding the conclusion that this algal oil is GRAS under the intended conditions of use.

Dietary Supplements. The term "dietary supplement," as defined in 21 USC 321(ff), means a product (other than tobacco) intended to supplement the diet that bears or contains a vitamin, mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any of the above ingredients. The definition further states that dietary supplement means products that are intended for ingestion and are not represented as a conventional food or as a sole item of a meal of the diet, and are labeled as a dietary supplement.

DHASCO-S is allowed as an article of trade as a dietary supplement in the U.S. under a Dietary Supplement Health and Education Act (DSHEA) notification. Pursuant to this act and consistent with the final regulations published by the FDA (see Table 11.1), a new dietary ingredient submission was made to the FDA for DHA oil derived from *Schizochytrium* sp. The FDA acknowledged receipt of the new dietary ingredient notification and did not respond with comment. The submission was placed on public display at Dockets Management Branch (70).

Europe

Novel foods are foods, food ingredients, and food production methods that have not been used for human consumption to a significant degree within the European

Community before May 15, 1997. Regulation EC No. 258/97 of January 27, 1997 of the European Parliament and the Council lays out detailed rules to authorize novel foods and novel food ingredients (see Table 11.1). Foods commercialized in at least one Member State before the entry into force of the Regulation on Novel Foods on May 15, 1997, are on the EU market under the "principle of mutual recognition." In order to ensure the highest level of protection of human health, novel foods must undergo a safety assessment before being placed on the EU market. Only those products considered to be safe for human consumption are authorized for marketing.

Companies that want to place a novel food on the EU market need to submit their application in accordance with Commission Recommendation No. 97/618/EC that concerns the scientific information and the safety assessment report required (see Table 11.1). Novel foods or novel food ingredients may follow a simplified procedure, only requiring notifications from the company, when they are considered by a national food assessment body as "substantially equivalent" to existing foods or food ingredients (in regard to their composition, nutritional value, metabolism, intended use and the level of undesirable substances contained therein).

Novel Foods

Foods, food ingredients, and productions methods are determined to be novel according to guidelines and usually in consultation with the competent authority in the Member State where the application is submitted. If a product is determined to be a novel food, the applicant prepares a dossier for submission and submits it to the Member State. The Member State has 90 days to review the dossier and provide an "opinion." The 90-day review process can take much longer depending on questions raised by the Member State undertaking the review and responses provided by the applicant. Assuming a favorable opinion is generated by the Member State conducting the review, the dossier is next passed on to the European Commission and to the other Member States, who have 60 days to raise "reasoned objections." If, during the course of the 60-day Member State review process, reasoned objections are raised and not resolved, the EFSA may be enlisted for an opinion. The EFSA serves as an independent point of reference for scientific opinion and may be requested to provide opinion by the Commission, the European Parliament, or the Member States. If there are no objections raised by Member States, the reasoned objections are satisfied by the applicant, or the EFSA offers a favorable opinion to counter the reasoned objections, then the product is approved, and a Commission Decision is passed by the Standing Committee on the Food Chain and Animal Health and published in the Official Journal of the European Communities.

Commission Regulation 1852/2001 (see Table 11.1) requires that the following information must be made public: name and address of the applicant; description allowing the identification of the food or food ingredient; intended use of the food or food ingredient; summary of the dossier, except for those parts for which the confidential character has been determined; and date of receipt of a complete request. The

Regulation also provides that the Commission must make the initial assessment report available to the public, except for any information identified as confidential.

Regulation 1852/2001 also lays down rules to protect information provided by applicants when requesting authorization under the Novel Foods Regulation. Pursuant to Regulation 1852/2001, Member States may not divulge information identified as confidential, with the exception of that information that needs to be made public in order to protect human health. Applicants, when submitting a novel food application, may indicate what information relating to the manufacturing process should be kept confidential on the grounds that its disclosure might harm their competitive position. This information must be duly justified, and it is then up to the competent authority, in consultation with the applicant, to decide which information will be kept confidential.

The "Substantial Equivalence" Concept

Article 3 of the Novel Foods Regulation sets the concept of "substantial equivalence" and provides that based on an opinion delivered by competent bodies if foods or food ingredients are "substantially equivalent" to "existing foods or food ingredients as regards their composition nutritional value, metabolism, intended use, and the level of undesirable substances contained therein," a simplified notification procedure applies. The concept of "substantial equivalence" embodies the idea that existing organisms or products used as foods or food sources can serve as a basis for comparison when assessing the safety and nutritional value of a food or food ingredient that has been modified or is new. Article 5 of the Novel Foods Regulation states that if a food or food ingredient has been determined to be substantially equivalent (Article 3), the applicant shall notify the Commission when the food or food ingredient is placed on the market. Applicants may market substantially equivalent food or food ingredients immediately after notification to the Commission; they do not have to wait for approval. The Commission is required to forward to Member States a copy of the notification and relevant details, if requested. Member States may oppose the marketing of such a product on their territory if it has "detailed grounds" of considering that the use of a food or a food ingredient endangers human health or the environment (see Article 12 of the Regulation). The Commission publishes a summary of those notifications in the "C" series of the Official Journal of the European Communities.

European Food Safety Authority

Following a series of food scares in the 1990s that undermined consumer confidence in the safety of the food chain, the EU concluded that it needed to establish a new scientific body charged with providing independent and objective advice on food safety issues associated with the food chain. Its primary objective would be to " . . . contribute to a high level of consumer health protection in the area of food safety, through which consumer confidence can be restored and maintained." The result was the creation of the EFSA. In May 2003, the five Scientific Committees providing the Commission with scientific advice on food safety were transferred to the EFSA. The

EFSA provides independent scientific advice on all matters linked to food and feed safety; it communicates to the public in an open and transparent way on all matters within its remit.

SCO Approved in the Europe Union

There are a number of SCO currently approved for use within the EU. DHASCO and ARASCO produced by Martek were commercialized in at least one Member State before the entry into force of the Regulation on Novel Foods on May 15, 1997, and are on the EU market under the "principle of mutual recognition." The Netherlands Ministry of Public Health, Welfare, and Sports granted exemption for the addition of ARASCO and DHASCO to preterm and term infant formula. This exemption was published in the State Journal on March 8, 1995 (71).

Martek has received approval for DHASCO-S. This SCO was the subject of novel foods approval and is currently marketed for select food applications as listed in the annex of the decision. This decision was published in the Official Journal of the European Communities (72).

Nutrinova notified the Commission in December 2003 of its intention to market a novel SCO product (DHA45 oil) obtained from the microalga *Ulkenia* sp., in accordance with Article 5 of the Novel Food Regulation (EC) 258/97. The German competent authority agreed with the company's claim that the product was "substantially equivalent" to the oil obtained from *Schizochytrium* sp. In December 2002, an applicant (Innovalg) notified the Commission of their intention to market a marine microalga *Odontella aurita* as a novel food, referring to an opinion on the "substantial equivalence" from the French competent authority. *O. aurita* is stated to be rich in the PUFA EPA. The product consists of dried algae and is intended to be used in a range of food products. The notifications for the microalga, *O. aurita* and DHA45 oil from *Ulkenia* sp. made under Article 5 of the Novel Food Regulation (EC) 258/97 are currently under review by other Member States and have not yet been linked to the Commission web site (see Notifications Pursuant to Article 5 of Regulation (EC) No. 258/97 of the European Parliament and of the Council (73).

Conclusion

The safety of SCO evaluated to date is based on several lines of evidence including: the inherent safety of the FA and other components of the oils, their presence in food (including human breast milk), the small quantities expected to be consumed, and knowledge of their metabolism; the absence of reports of pathogenicity or toxigenicity of the source organisms used for their production; published results of nonclinical safety studies in rodent and nonrodent species demonstrating no unexpected, treatment-related, dose-dependent, adverse toxicological effects; human clinical studies in target populations monitoring safety outcomes and documenting no serious treatment-related adverse effects; and historical safe use of the products, including use in infant formulas (preterm and term), as dietary supplements and as food ingredients.

In the U.S., the FDA has the primary responsibility to regulate new food ingredients, including SCO used as food ingredients. Manufacturers may propose the addition of new food ingredients by either filing a food-additive petition with the FDA to request a formal premarket review, or making a GRAS determination. The GRAS notification process has become a popular choice not only for introducing new food ingredients, but specifically for several new SCO used in infant formulas and food applications. Manufacturers wishing to obtain approval to use a new ingredient in the EU traditionally approach the competent authority of a Member State to consult on the appropriate (e.g., novel food application) approach to obtain premarket clearance. The Novel Foods Regulation provides two routes to authorize novel foods, a full procedure and a simplified procedure based on the concept of "substantial equivalence." Under Article 4 (the "full procedure"), an initial assessment from one Member State is made and circulated for formal review by the competent authorities in the other Member States. If Member States are not unanimous at this stage, a decision is taken by majority vote. The EFSA may be asked to advise on any concerns related to the risk assessment before a vote is taken. Under Article 5 (the "simplified procedure"), an applicant can apply to a Member State for an opinion on "substantial equivalence," this is then forwarded to the European Commission along with a notification of the applicant's intention to market the product.

In the U.S., DHASCO and DHASCO-S have been marketed as dietary supplements. DHASCO-S oil was the subject of a New Dietary Premarket Notification submitted to FDA under the provisions of the DSHEA. The use of DHASCO and ARASCO in infant formulas has been determined to be GRAS by scientific procedures after examination by qualified experts. This conclusion was reviewed by the FDA as part of GRAS notification no. GRN 000041 and GRN 000080, with no objections. Commercial infant formulas (preterm and term) containing DHASCO and ARASCO have been sold in the U.S. and in over 60 countries worldwide, including European countries such as Belgium, Finland, France, Greece, The Netherlands, Portugal, Spain, Turkey, and the United Kingdom. The use of DHASCO-S as a nutritional food ingredient has been determined to be GRAS by scientific procedures following review by qualified experts. This conclusion was reviewed by the FDA as part of GRAS notification no. GRN 000137, with no objections. DHASCO-S is also approved for use as a novel food ingredient in the EU.

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Chapter 12

Nutritional Aspects of Single Cell Oils: Uses and Applications of Arachidonic Acid and Docosahexaenoic Acid Oils

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Introduction

One of the driving forces for the development of single cell oil (SCO) containing long-chain polyunsaturated fatty acids (LCPUFA) was the presence in human milk of two particular LCPUFA, docosahexaenoic acid (DHA) and arachidonic acid (AA). Until recently these polyunsaturated fatty acids (PUFA) have not been added to infant formulas. Once it was recognized that these two PUFA played an important role in the brain, attempts were made to provide these PUFA naturally from fish oils and egg phospholipids. It was relatively easy to obtain DHA from oils such as tuna oil (1); providing AA was more difficult. When it was found that AA-containing oils were produced by certain species of soil fungi (2), research soon established that it was possible to harvest this oil in commercial quantities. Similarly, a DHA-containing oil from a marine microalgae was used to produce commercial quantities of DHA (3).

Since the brain is rich in LCPUFA, it is important to understand the role of these fatty acids (FA) in brain function. The brain has the second highest concentration of lipids in the body, after adipose tissue, with 36–60% of the nervous tissue being lipids (4). The lipids in the brain are complex lipids and include glycerophospholipids (GPL), sphingolipids (sphingomyelin and cerebrosides), gangliosides, and cholesterol with little or no triglycerides and cholesterol esters (5). Brain GPL contain a high proportions of LCPUFA, mainly DHA, AA, and docosatetraenoic acid (C22:4n-6), with very small amounts of α-linolenic acid (ALA) and linoleic acid (LA). The proportion of DHA and AA in the GPL of brain gray matter is higher than the white matter (6,7), with phosphatidylethanolamine (PE) and phosphatidylserine (PS) containing the most DHA of all the GPL, while PE and PI contain the highest proportions of AA. The DHA plus AA content of the adult cerebral cortex is approximately 6% dry wt and 2% of the white matter (6). The n-6 content (20:4n-6 plus 22:4n-6) of the cerebral cortex is similar to that of the DHA level and in white matter there is a higher proportion

of n-6 than n-3 PUFA (6). The highest proportion of DHA in membrane lipids is found in the disk membranes of the rod outer segments of photoreceptor cells in the retina (8,9). Carrie *et al.* (10) showed that the proportion of DHA in 11 different regions of the rat brain varied from 7% GPL FA in the pituitary gland to 22% in the frontal cortex. The variation in the proportion of AA ranged from 5% in the pons medulla to 18% in the pituitary gland. This gland was the only region where the proportion of AA exceeded that of DHA.

DHA and AA are present in other tissues in the body but in lower proportions. For example, in the guinea pig the proportion of DHA of all tissues except neural tissue was <0.5% total tissue FA, while in whole brain it was 6–7% total fatty acids (TFA) (11). On a whole body basis, the brain contained approximately 22–25% of the total DHA in the body, with approximately 50% of the DHA being in the carcass (muscle and adipose tissue). The same study showed that the AA was mostly distributed in the carcass (70%), with only 2% in the brain.

The high levels of DHA and AA in the brain gray matter of over 30 different mammalian species (Table 12.1) (12), led to early speculations that these PUFA play a crucial role in the nervous system. In the 1970s, the n-6 PUFA were regarded as essential for humans, while the n-3 PUFA were only thought to be essential for fish and other marine species. The first clue for a physiological role of n-3 FA in mammals came when it was reported that dietary n-3 PUFA fed to rats led to nearly double the response of the retina to visual stimulation of rats compared with when n-6 PUFA were fed (13). Since then, intensive study of the role of DHA in the brain revealed that it plays a vital role in many different parts of the brain, the most obvious role being related to membrane function. In summary, DHA plays a crucial role in membrane-related events (membrane order that can influence the function of membrane receptors such as rhodopsin) (14,15); regulation of dopaminergic and serotoninergic neurotransmission (16); regulation of membrane-bound enzymes (Na/K-dependent ATPase) (17); signal transduction *via* effects on inositol phos-

TABLE 12.1
Polyunsaturated Fatty Acids (FA) in Ethanolamine Phosphoglycerides of Mammalian Liver and Brain Gray Matter (mg/g total FA and aldehydes)[a]

FA	Brain	Liver
18:2n-6	12 (3–24)[a]	120 (31–470)
20:3n-6	7 (2–10)	11 (3–45)
20:4n-6	120 (89–150)	130 (41–210)
22:4n-6	63 (42–80)	10 (1–56)
22:5n-6	12 (2–29)	5 (1–14)
18:3n-3	5 (1–10)	21 (1–54)
20:5n-3	6 (1–12)	23 (5–78)
22:5n-3	7 (3-19)	54 (3–110)
22:6n-3	220 (160–290)	98 (2–220)

[a]Results are shown as the mean value and range for 25 species. *Source:* Reference 12.

phates, diacylglycerol, and protein kinase C (18); alteration of ion flux through voltage-gated K^+ and Na^+ channels (19,20). In addition, DHA is involved in metabolic events (regulation of the synthesis of eicosanoids derived from AA) (21); as a precursor of docosatrienes and 17S resolvins (novel anti-inflammatory mediators) derived from DHA (Fig. 12.1) (22), and in gene expression (regulation of gene expression of many different genes in rat brain in short- and long-term studies) (23–28). Finally, DHA is involved in cellular events such as regulation of phosphatidyl serine levels (29) that appears to be involved in the protection of neural cells from apoptotic death (30); stimulation of neurite outgrowth in PC-12 brain or neuron cells (31,32); selective accumulation of DHA by synaptic growth cones during neuronal development (31,32); regulation of neuron size (33,34); regulation of nerve growth factor (35); and as a precursor of neuroprostanes (DHA oxidation products) (Fig. 12.1) (36,37).

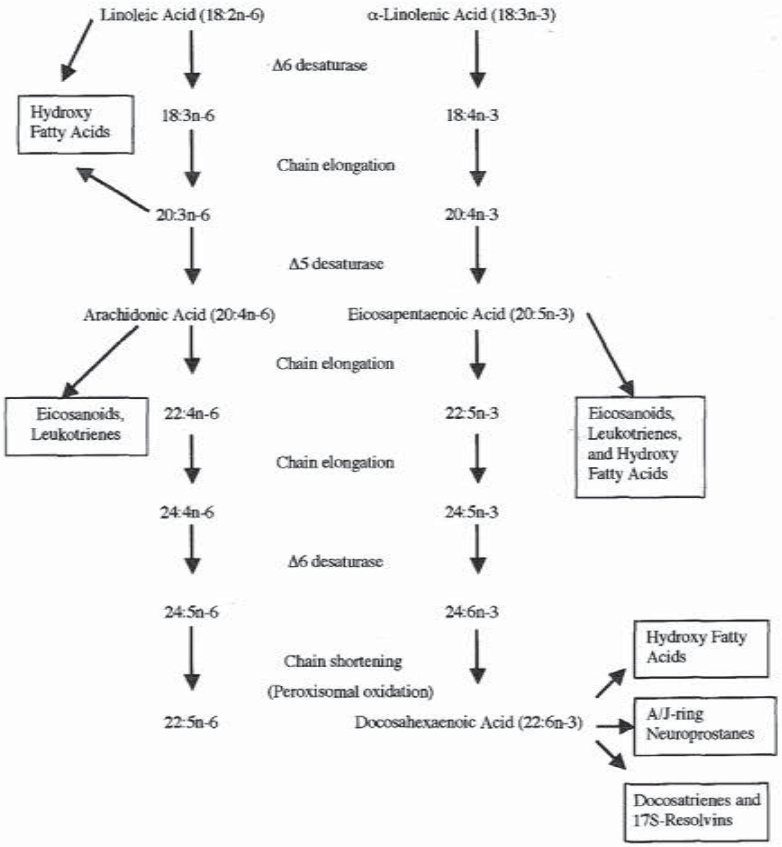


Fig. 12.1. Metabolic pathway involved in converting essential fatty acids into their longer chain metabolites and other products.

AA is the predominant n-6 PUFA in mammalian brain and neural tissue (Table 12.1) and, like DHA, is found in *sn*-2 position of the glycerol backbone of membrane GPL. AA, therefore, plays a key role in membrane function. The release of AA from the membrane GPL is due to the receptor-mediated activation of phospholipase A_2 or phospholipase C and diacylglycerol lipase (38). Once released, AA can become a substrate for oxidative enzymes, such as cyclooxygenases (COX-1, COX-2), lipoxygenase, or cytochrome P450 monoxygenases, that convert it to a number of bioactive eicosanoids like prostaglandins, prostacyclins, thromboxanes, and leukotrienes (Fig. 12.1) (39,40). The functional role of AA appears to be mediated either by the FA itself or through the bioactive metabolites produced by the oxidative reactions. For example, AA exerts diverse actions on acetylcholine receptors such as a short-term depression by blocking of the receptor and long-lasting potentiation by activation of protein kinase C pathway (41). Furthermore, synaptic activation of glutamate receptors has been reported to release AA. This suggests a role in synaptic transmission (42,43).

Abnormalities in AA metabolism in brain have been linked to a number of brain disorders such as bipolar disorder (44), Alzheimer's disease (45), schizophrenia (46), and ischemia (47). COX-2, an enzyme that converts AA to eicosanoids, is highly expressed in different regions of the brain such as hippocampus, cortex, and amygdala (48). Age-dependent cognitive deficits and neuronal apoptosis have been reported in transgenic mice over expressing COX-2 with a concurrent increase in prostaglandin levels in the brain (49), suggesting that neuronal COX-2 may contribute to the pathophysiology of age-related diseases. The reduced skin flushing response to niacin in schizophrenic subjects has been known for years; since the primary mechanism is conversion of AA to prostaglandin D2, this also suggests an abnormal AA metabolism in these subjects (50). Treatment of schizophrenic patients with a 2 g dose of ethyl eicosapentaenoate acid (EPA, 20:5n-3) leads to a significant improvement of the condition with an elevated level of AA in erythrocyte FA (46); it is speculated this may result from the inhibition of phospholipase A_2 by EPA (46).

There is a rapid increase in the weight of the human brain postnatally, until the infant is about two years old. Associated with this, there is a rapid accretion of DHA and AA in the infant brain during the first postnatal year (51). It is thought that the DHA and AA for brain growth are largely derived from mothers' milk. Breast feeding provides at least 49 mg of DHA and 93 mg of AA to the infant each day depending on the PUFA level in the mother's milk (52). It is known that milk LCPUFA levels can be influenced by diet; usually the LCPUFA are in the range from 0.2 to 1.0% of total milk FA (53).

The fetal brain is believed to be able to produce a limited amount of DHA from ALA; the liver may also be able to produce some DHA (Fig. 12.1) (54), but it is believed that this is insufficient for optimal development (55). It has been argued that based on the rate of accretion of DHA into the human brain, there is a need to supply

DHA *via* breast milk or infant formula for at least the first six months of life (56). There has been little discussion about the capacity of newborn infants to synthesize sufficient AA for brain growth. The limited capacity of the neonatal infant to synthesize LCPUFA has been a driving force in developing infant formulas containing these FA.

Another rationale as to why it is necessary to add LCPUFA to infant formulas is based on the decline in blood LCPUFA levels after birth. Blood levels of DHA and AA in infants fed standard formulas are lower than those of breast-fed infants (57–60). Formulas containing LCPUFA can increase blood LCPUFA levels so they more closely resemble those found in breast-fed infants (61,62). Consistent with the decline in blood levels of DHA and AA in formula-fed children, two studies have examined brain PUFA levels in formula-fed and breast-fed infants. Both found that there was a significantly lower level of DHA, but not AA, in the brain tissue of children who had been mainly fed on infant formulas (lacking DHA and AA) (63,64).

These studies underpin the discussion that has been conducted throughout the world on the importance of adding DHA and AA to infant formulas. Before such action was undertaken, many studies were conducted in animals and primates.

SCO Studies in Animals: PUFA Levels in Tissues and Functional Studies

Many studies conducted on SCO in animals have been concerned with the efficiency of these oils in supplying tissues with PUFA, especially in relation to brain PUFA and brain function. This section will discuss several examples from the literature that illustrate that LCPUFA from SCO sources are bioavailable and can influence physiological function. Other studies have been concerned with the safety of these oils, and this is dealt with on page 188.

Ward *et al.* (65) studied the effect of adding LCPUFA from SCO on the brain and red blood cell FA composition. Rat pups were reared artificially from day 5 to day 18, postnatally, using a gastromy tube. The study compared three levels of DHA and three levels of AA in a factorial design. The basal diet contained LA and ALA and no LCPUFA. The results showed that supplementing the formula with AA or DHA during the period of brain development increased deposition of these PUFA in the brain and red blood cells. Furthermore, it was found that increasing levels of each PUFA affected the levels of the other PUFA (the highest dietary AA decreased tissue DHA levels and vice versa).

Abedin *et al.* (66) compared the efficiency of ALA versus DHA in contributing DHA to various tissues (liver, heart, retina, and brain) in guinea pigs. In this study, LCPUFA from SCO were fed to guinea pigs from 3 weeks until 15 weeks of age. The LA content in the diets was constant (17% TFA) with the ALA content varying from 0.05% (diet S), to 1% (diet A), and to 7% (diet C). Diet A had an LA:ALA ratio of 17.5:1 and was structured to closely replicate the principal LCPUFA found in human breast milk (0.9% AA and 0.6% DHA). In the retina and brain phospholipids, the high ALA diet (diet C) or dietary DHA supplementation produced moderate increases in DHA levels compared with diet S (low ALA diet). There was no change in retinal or brain AA following dietary AA supplementation. This was in contrast to the liver and heart in which the dietary DHA and AA supplement led to large increases (up to 10-fold) in the tissue levels of these PUFA. The data confirmed that dietary ALA was less effective than dietary DHA supplementation (on a g/g basis) in increasing tissue DHA levels, and that tissues vary greatly in their response to exogenous AA and DHA; the levels of these long-chain metabolites is most resistant to change in the retina and brain compared with the liver and heart.

A recent study in rhesus monkey neonates examined the effect of inclusion of DHA and AA in the rearing infant formula on neuromotor development (67). In this study, 28 nursery-reared rhesus macaque infants were divided into two groups, one of which was fed a formula with DHA (1% of the fat) and AA (1% of the fat) from SCO sources; the other group was fed the standard formula that was devoid of the LCPUFA. Neurobehavioral tests were conducted weekly from day 7 of life through day 30. Plasma DHA and AA concentrations in the supplemented group were significantly higher than the control group at 4 weeks of age. The monkeys fed the supplemented formula showed stronger orienting and motor skills than those fed the standard formula; the most pronounced differences were at day 7 and 14. Supplementation with LCPUFA had no influence on temperament. These data support the inclusion of LCPUFA in infant formulas for optimal development.

Some studies have compared the effects of diets with DHA alone versus those with DHA plus AA. One such study was conducted by Auestad *et al.* (68), in which they measured the auditory brainstem-evoked response (ABR). In previous studies, it had been found that juvenile offspring of rats fed high-DHA diets through gestation and lactation had a longer ABR that was associated with higher DHA and lower AA proportion in the brain (69). In the Auestad *et al.* study (68), the ABR was assessed in juvenile rats fed high-DHA diets postnatally and compared with diets containing both DHA and AA. It was found that the DHA and AA levels in the brain increased with supplementation. In contrast to the earlier study, this study found that ABR was shorter in the high-DHA group than the DHA plus AA group and not different from the unsupplemented or dam-reared suckling group. Clearly, further studies are needed to understand the relationship between dietary DHA and the development of the auditory system over a range of DHA intakes and discrete periods of development.

Blanaru *et al.* (70) examined the effect of an increasing dose of AA (0.3 to 0.75% fat) with a constant DHA level (0.1% fat) from SCO sources on bone mass in piglets in a study starting at day 5 and finishing at day 20 after birth. The study was initiated due to an upsurge in interest in the effect of PUFA on bone biology (71). Bone modeling was unaffected by the different treatments, but the whole body bone mineral content was elevated in piglets fed 0.6 and 0.75% AA. The effect of altering the dietary intake of DHA in this model is not known.

Some studies have compared the effects of different sources of LCPUFA on various outcomes in animals. For example, Mathews *et al.* (72) compared SCO sources of DHA and AA with egg phospholipids as a source of these PUFA on overall animal

health and safety. In this study, piglets consumed a skim milk formula from day 1 until day 16 after birth. The formulas with LCPUFA provided 0.3 and 0.6 g/100 g TFA as DHA and AA, respectively. The control group contained no DHA or AA. There was no difference in gross liver histology between the groups; the apparent dry matter digestibility was 10% greater in the SCO and control groups than in the group fed egg phospholipid PUFA. The plasma DHA proportion was higher in the SCO group than in the egg phospholipids group, while the plasma AA proportion was higher in the SCO group than the control group. In summary, these studies revealed that LCPUFA from SCO sources were bioavailable and that they had the capacity to alter physiological function in small animals and primates.

Safety Aspects of SCO

The main concern about SCO derived from algae and fungi has been that they are new food ingredients without a history of safe use in infant feeding anywhere in the world. This has meant that these oils have had to undergo extensive toxicological testing in various animal species. The results have been favorable (73–79), and authorities in several countries have approved their use in infant formulas. In the U.S., the Food and Drug Administration (FDA) has given generally recognized as safe status to SCO, thus permitting their use in infant formulas (80).

SCO Studies in Infants

Following the successful trials on bioavailability and safety of SCO in animals and primates, there have been a number of trials in term and preterm infants that have included LCPUFA from various sources into infant formulas. The authors have identified twelve randomized clinical trials (RCT) designed to test the efficacy and safety of adding either n-3 LCPUFA (DHA and EPA) or a combination of DHA and AA to formulas for term infants, that have been published in full. Four of these studies used SCO (62,81–84), while the remainder used other lipid sources (61,85–94). It is doubtful whether the source of LCPUFA has much effect on LCPUFA status of the infant (95).

Trial Design and Treatments

The four trials involved healthy term infants fed formulas from near birth and all but one had a breast-fed reference group. Most trials appeared to have adequate randomization and masking procedures, and most presented power calculations for their primary outcome measurements. Therefore, the trials involving term infants are generally of good methodological quality.

The levels of n-3 LCPUFA used in the trials ranged from 0.1 to 1% total fat while the AA ranged from 0.4 to 0.7% TFA. Four trials assessed the effect of supplementation with n-3 LCPUFA with no AA (61,85,89,92).

Outcomes

Benefits of adding LCPUFA to formulas on visual acuity assessed by both visual evoked potential or Teller cards have been reported in some studies (81), while other studies have shown no difference between LCPUFA supplemented and unsupplemented infants (85). A systematic review of three trials on visual acuity in term infants indicated an improvement in card acuity with LCPUFA treatment at two months of age only (96). There is also mixed evidence for the support of an effect of dietary LCPUFA on more global measures of development (Bayley Scales of Infant Development). Birch *et al.* (82) have reported benefits of dietary LCPUFA; however, the larger studies conducted by Scott *et al.* (86) and Auestad *et al.* (62) showed no effect of LCPUFA supplementation on the Bayley Scales of Infant Development. Possible interpretations of these data include a small individual effect, or that only a proportion of infants will benefit, or the presence of confounding variables. Further studies are needed to elucidate this issue.

There have been no negative findings in relation to growth in term infants regarding LCPUFA supplementation of infant formulas. This is despite the fact that four trials have supplemented formulas with DHA alone without added AA for periods of up to one year, resulting in the AA status of infants being depleted. Therefore, there is no evidence that n-3 LCPUFA supplementation of term infant formulas causes perturbations of growth.

Trials Involving LCPUFA in Preterm Infants

The last trimester of pregnancy is the time when DHA accretion in the brain and nervous system is at its greatest velocity. Therefore, many preterm infants, especially those born before 30 weeks, are born with negligible body stores of DHA; subsequently they are fed with milks that contain no DHA or levels that are much lower than what these infants would have received if they were still *in utero*. Preterm infants are more at risk than term infants of disturbed DHA accumulation and thus may have the most to gain from DHA supplementation.

The authors are aware of 13 RCT reported in at least 20 separate papers. Four of these studies used SCO (97–102), while the remainder used lipids from other sources (103–116). These trials were designed to test the efficacy and safety of varying levels of DHA, EPA, and AA in the diets of preterm infants.

Trial Design and Treatments

All the trials reported adequate concealment of allocation and in general their methodological quality is more robust than earlier trials.

Outcomes

The original trials that showed a benefit on electroretinographic responses and visual acuity all supplemented with fish oil. Of the SCO trials, only that of O'Connor *et al.*

(100) assessed visual function and showed benefit to improving visual evoked potential (VEP) acuity but not Teller card acuity. Of the three trials that have assessed global development, one reported an advantage of LCPUFA supplementation on psychomotor development in infants born at less than 1250 g (100). The data from O'Connor *et al.* (100) suggested that the more immature and sick infants may have the most to gain from LCPUFA supplementation; this highlights the notion that some subgroups may be more sensitive to the effects of LCPUFA. Further work is needed to best maximize the potential benefits on early childhood development.

Although most trials show that there is no effect of LCPUFA supplementation on growth, a recent trial suggested an enhancement of growth (99). Two separate systematic reviews and meta-analyses combining growth data from all published RCT showed no difference in any growth parameter between supplemented and unsupplemented infants (117,118). Although the outcome data from the individual LCPUFA intervention trials with visual outcomes consistently indicated beneficial effects of LCPUFA supplementation, the two available systematic reviews/meta-analyses of these data have not been complete (118,119). One review could not combine the visual outcome data because of different assessments and methodologies and differing assessment times (118). The other review included data from three randomized trials and one non-randomized study and concluded that there was a beneficial effect of LCPUFA treatment on visual acuity at two and four months corrected age (119). Thus, despite the promising beneficial effect of DHA supplementation on the neural outcomes of preterm infants, trials with standard methodologies and follow-up of infants beyond 12 months corrected age are necessary to more precisely assess the extent of benefit offered by LCPUFA supplementation.

SCO Studies in Adults

There have been relatively few studies on SCO in adults. Nelson and colleagues conducted two separate studies that involved feeding a small group of volunteers with either DHA or AA derived from SCO. These studies led to a number of papers by the group, published in the period 1997–1999 (120–128).

The aim of the DHA study was to examine the effects of feeding DHA-rich triacylglycerol (TAG) on the FA composition, eicosanoid production, select activities of human peripheral blood mononuclear cells, plasma lipoprotein concentration, and the FA composition of plasma lipids and adipose tissue. The 120-d study with 11 healthy men was conducted at the Metabolic Research Unit of Western Human Nutrition Research Center. Four subjects (control group) were fed the stabilization diet or basal diet (15, 30, and 55% energy from protein, fat, and carbohydrate, respectively) throughout the study; the remaining seven subjects were fed the basal diet for the first 30 d, followed by 6 g DHA/d for the next 90 d. DHA replaced an equivalent amount of LA; the two diets were comparable in their total fat and all other nutrients. The ratio of saturated plus *trans* FA to monounsaturated FA to PUFA in the diets was 10:10:10. Both diets were supplemented with 20 mg D α-tocopherol acetate/d.

The white blood cell FA composition, eicosanoid production, immune cell functions, plasma lipoprotein concentrations, and the plasma and adipose tissue FA composition were examined on day 30 and 120. There was an increase in white blood cell DHA from 2.3 to 7.4% and a decrease in the proportion of AA from 19.8 to 10.7%. There was also a lowered prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) production by 60–75%, in response to lipopolysaccharide. Natural killer cell activity and *in vitro* secretion of interleukin-1β and tumor necrosis factor α were significantly reduced by DHA feeding (120,121). The concentration of plasma cholesterol, low-density lipoprotein and apolipoproteins [A_1, B, and lipoprotein (a)] were unchanged after 90 d, but the TAG levels were significantly reduced and the high-density lipoprotein-C and apolipoprotein-E levels were increased significantly. The proportion of plasma DHA rose from 1.8 to 8.1% after 90 d on the high-DHA diet. Interestingly, the plasma EPA levels rose from 0.4 to 3.4% in subjects on the high-DHA diet, despite the diet being devoid of EPA. The DHA proportion in adipose tissue rose significantly from 0.1 to 0.3%, but the amount of EPA did not change (122). The effects of the high-DHA diet on platelet aggregation were also studied in this experiment; no significant effects were found in blood coagulation parameters, platelet function or thrombotic tendency (122,123).

These authors also studied the effect of the DHA-rich diet on the conversion of deuterium-labelled 18:2n-6 and 18:3n-3 to LCPUFA. The labeled compounds were administered as TAG at the end of the DHA-feeding period and blood samples were taken over the following 72 h. The DHA supplementation significantly reduced the concentrations of most deuterium-labeled n-6 and n-3 LCPUFA metabolites in plasma lipids. For example, the accumulation of deuterium-labeled 20:5n-3 and 22:6n-3 was depressed by 76 and 88%, respectively. The accumulation of deuterium-labeled 20:3n-6 and 20:4n-6 also decreased by 72% for both PUFA (124). These authors calculated that the accumulation of n-3 LCPUFA metabolites synthesized from 18:3n-3 would be reduced from about 120 mg/d to 30 mg/d by supplementation with 6.5 g DHA/d. It was calculated that the accumulation of n-6 LCPUFA metabolites, synthesized from 18:2n-6, would be reduced from about 800 mg/d to 180 mg/d. The authors suggested that health benefits associated with this level of DHA supplementation would be the result of reduced accretion of n-6 LCPUFA and an increase in n-3 LCPUFA levels in tissue lipids.

A study on AA was conducted by Nelson *et al.* (125). In this study, 10 healthy men lived at the Metabolic Research Unit for 130 d. All subjects were fed a basal diet containing 27 energy percentage (en%) fat, 57 en% carbohydrate, 16 en% protein, and 200 mg AA/d for the first and last 15 d of the study. Additional AA (1.5 g/d) was incorporated into the diet of six men from day 16 to 65 while the remaining four subjects continued to eat the basal diet. The ratio of saturated plus *trans* FA to monounsaturated FA to PUFA in the diets was 7:10:7. The diets of the two groups were crossed over from day 66 to 115. Dietary AA had no significant effect on the blood cholesterol levels, lipoprotein distribution, or apoprotein levels.

The plasma TFA composition was markedly enriched in AA after 50 d ($P < 0.005$). The AA proportion in plasma PL increased from 10.3 on the basal diet to

19.0% after the AA-enriched diet. There was a significant rise in the proportion of AA in the red blood cells that mainly replaced LA. The adipose tissue FA composition was not influenced by the AA-enriched diet (125). Platelet aggregation in the platelet-rich plasma was determined using ADP, collagen, and AA. There were no significant differences in platelet aggregation before and after consuming the AA-enriched diet (126). There were no significant changes in any of the indices of blood clotting (prothrombin time, partial thromboplastin time, antithrombin III levels, and *in vivo* bleeding times) between diet groups. Surprisingly, there was only a small change in platelet AA proportion during the AA feeding period. The *in vitro* secretion of LTB_4 and PGE_2, from *in vitro* stimulated white blood cells, was significantly increased after the AA-enriched diet, but it did not alter the secretion of tumor necrosis factor α, interleukins-1 β, -2, -6; and the receptor for interleukin-2 (127). At the end of each diet phase, each subject was dosed with about 3.5 g of deuterium-labeled 18:2n-6 as TAG. The concentration of deuterium-labeled 20:3n-6 and 20:4n-6 were both 48% lower ($P < 0.05$) in plasma lipids from the AA-enriched group compared with the low AA diet group (128).

Conclusion

The commercial development of SCO and their FDA approval has allowed the addition of LCPUFA from SCO sources to infant formulas. This advance has enabled the composition of infant formulas to approach that of human milk, a goal that is sought by both formula companies and parents. Future research on SCO should examine the nutritional benefits of SCO in adults.

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106

Chapter 13

Down-Stream Processing, Extraction, and Purification of Single Cell Oils

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General Considerations for Single Cell Oil Extraction

In all cases, whether one is dealing with yeasts, molds (fungi), or algae, there is the common problem of biomass recovery from the fermentor and subsequent extraction of the oil. Given that all the single cell oils (SCO) that will be discussed in this chapter are ones rich in a particular polyunsaturated fatty acid (PUFA), then there is an obvious need to avoid sustained high temperatures or other conditions that could lead to the oxidation and rancidity of the oils during extraction. Also, since SCO are of very recent origin, their extraction initially caused some concern that novel processes might have to be developed that would add significantly to the overall cost of manufacture. However, extraction of SCO proved to be relatively simple and no new extraction machinery had to be built to accommodate the microscopic cells of microorganisms; also, there was no need to develop new solvent extraction methods. In practice, those skilled in the art of oil extraction found that oil extraction from microorganisms was no more difficult than extracting oil out of other sources of valuable oils.

In a typical SCO process (1) using a non-photosynthetic organism, the average fermentor would be of about 100,000 L (100 m^3) capacity and this could be expected to yield between 50 and 100 kg biomass/m^3. Thus at the end of a fermentation run, that typically might last for 4–6 d, there are 100,000 L of culture broth to be processed from which the biomass has to be separated from the liquor. Separation is sometimes performed by centrifugation though more usually rotary vacuum filtration or direct filtration is used. The spent liquor is of no value, though it will probably require some processing before it can be safely discharged into a river or water system in order to satisfy the local environmental regulations. The still wet biomass contains the oil; at this stage it may typically still contain 80% water. The mass may be on the order of 25 T (~25 m^3)—5 T dry cells with 20 T water. This has to be quickly dried to a point at which the oil is stabilized. Unless the biomass has been pretreated in some way, it contains many enzymes that continue to be active; some, such as lipases and esterases, are activated by the very process of removing the cells from their source of nutrients.

Under certain conditions, the cells may therefore start to consume the very oils that they have accumulated.

Once the biomass has been stabilized and the enzymes inactivated, usually by heating, the water can be removed by further pressure filtration followed, if necessary, by drying, often using a spray dryer. The final preparation of dry cells in a powder form is then available for oil extraction. However, because all of the enzymes that could degrade the oil have been heat-inactivated, the cells can be stored for several weeks before they need be extracted.

Solvent extraction using hexane is the preferred means of obtaining the oil. Extraction equipment used by the industry to obtain oils from small amounts of plant material (e.g., some exotic oils and the essential oils) can be used without adaptation since the microbial biomass at this stage approximates to dried and powdered plant material. The large-scale equipment used by oil extractors to recover oils from the commodity plant sources (soybeans, sunflower seeds, rapeseed, corn, etc.) are too large to be used on the relatively small quantities of microbial biomass that have to be extracted. However, smaller-scale equipment that can handle 10–50 T of material/d is appropriate. Operators of such equipment have not found any intrinsic difficulties in handling microbial biomass for oil recovery. Further refinement of the oil (see Fig. 13.1) to clean it and remove phospholipids and various nonsaponifiable materials is carried out to produce the final, usually clear bright, oil. These final processes are performed on all oils destined for direct consumption and, consequently, the same procedures and equipment that are used for plant oils are also used for the SCO.

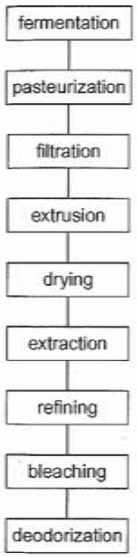


Fig. 13.1. Overview of industrial process operations for extraction of single cell oil.

One final point that should be stressed is that many microbial oils contain relatively large quantities of their own antioxidants; this means that the SCO have been found to be much more stable than corresponding oils from plants and marine animals. This is a particularly useful attribrute during the initial steps of oil extraction. The standard commercial practice, however, is to add further antioxidants to the oils in order to assure the customer that all the necessary steps have been taken to ensure complete stability of the oil.

Oil Extraction Processes

Extraction of γ-Linolenic Acid SCO from Mucor circinelloides

The first commercially viable SCO process was the production of an oil rich in γ-linolenic acid (GLA) using *Mucor circinelloides* (2,3). This was produced by J. & E. Sturge, Selby, North Yorkshire, UK, whose core business was citric acid production using *Aspergillus niger*. The oil was sold under the name Oil of Javanicus and was available from 1985 to 1990; the process eventually closed because of the advent of cheaper alternative sources of GLA oils, namely borage oil (also known as Starflower oil) and evening primrose oil.

The GLA-SCO process was run in one of the existing 220 m^3 fermentors that was normally used for citric acid production (3); this gave a yield of about 50 kg dry biomass/m^3 and an oil content of 25%, within 72–90 h. The physical removal of the biomass from the fermentor and its separation from the culture medium took over 6 h.

Examination of the very first batch of oil extracted from *M. circinelloides* indicated the presence of free fatty acids (FFA) at about 3–5% of the total oil, which then contributed certain undesirable characteristics to the oil. These FFA were quickly realized to be artifacts of the down-stream processing system since they were not present if the oil was quickly extracted from small samples of the biomass taken from laboratory-level fermentors. Clearly, the microbial cells were metabolically active: at the time of harvesting, all the glucose in the culture medium had been consumed—indeed this was the desired end-point of each fermentation run; this then caused the cells to activate their own lipases and phospholipases to mobilize the oil that had accumulated in the fermentor. The cells, after all, were physically starving and considered it was time to consume the oil reserves they had laid down in order to ensure their own continued viability.

The solution to this unwanted activity of lipases that were degrading the oil and resulting in the formation of FFA was to heat the broth in the fermentor in the final stages of the run. In practice, because fermentation processes are exothermic, the simple solution was to switch off the cooling system and allow the fermentor to heat up to 55–60°C and hold it at this temperature for at least 30 min prior to harvesting. When this procedure was put into practice, the presence of the FFA in the final oil was negligible and the oil met all subsequent tests for its stability, safety, and suitability for use as a dietary supplement.

The biomass, once heat-stabilized, was de-watered using filtration and drying; it was then passed to a company (Bush Boake & Allen, Long Melford, UK) that specialized in the extraction of essential (terpenoid-type) oils from various plant materials. The equipment that was used was unmodified from that in daily use by the company and had the advantage of being able to handle the relatively small volumes of biomass that were available. Conventional, commercial oil extraction from plant seeds uses huge extraction units that can deal with tens, if not hundreds, of tons of material per hour and would be entirely inappropriate for handling the small amount of fungal biomass available at any one time. The volume of oil that is "held up" in such systems is often of the order of 100 T; this would mean that 5 T of GLA oil would be simply "lost" in one of these units—or at the very least heavily contaminated with the oil already in the system.

Over 98% of the oil within the cells of *M. circinelloides* could be extracted directly using hexane in one of the speciality extractors. The crude oil, however, required further processing to remove the nonsaponifiable materials—mainly phospholipids, some sterols, and nonlipid components that had co-extracted into the oil itself. Standard oil refining procedures, including deodorization as practiced by the oil industry, were successfully applied to the crude oil extract; this generated a bright, pale yellow oil. However, small pilot-scale equipment had to be found that could handle the small volumes of oil involved. For this part of the work, pilot-scale equipment used by Simon Rosedown Ltd (now DeSmets), Hull, UK, for trial runs on small amounts of unusual plant oils, was commissioned. Once more, the oil was purified to a high level using existing equipment with no need for any modification.

To all intents and purposes, oil extraction from dry fungal biomass posed no significant problem provided that it was performed in equipment of an appropriate size. Once the oil had been extracted, its further refinement and purification followed conventional procedures and again posed no problems. The specifications of the oil are given in Table 13.1. Although an antioxidant was added to the final oil, this was merely a precaution since the oil itself was highly stable to oxidation as, evidently, the natural antioxidants within the fungal cells had been co-extracted with the oil. Because the production organism has had a long historical association with traditional fermented foods of tempeh and tapé, originally known as *Mucor javanicus* (indicating its oriental origins in Java), this was an important factor in the oil being given generally recognized as safe (GRAS) status, although additional feeding trials with animals were, of course, carried out as well.

The *Mucor* process ceased production in 1990 due to competition with borage oil; Borage oil contained slightly higher levels of GLA (~22%) but it could be produced at a cheap (though subsidized) price. Over the six years of production, about 50–60 tons of GLA-SCO were produced. The oil had excellent long-term stability and, even without the addition of antioxidant, showed little or no deterioration in its GLA content over at least 10 years storage at room temperature, in air and in sunlight. Although the commercial viability of this oil was short-lived, it nevertheless demonstrated for the first time that SCO production was achievable, and that the oil itself was equal or better than the best plant oils in its safety and lack of toxicity. A more complete account of this process has been given elsewhere (2).

TABLE 13.1
Specifications of the γ-Linolenic Acid Single Cell Oil from *Mucor circinelloides*[a]

Oil	
Appearance	Pale yellow, clean, and bright
Specific gravity	0.92 at 20°C
Peroxide value (meq/kg oil)	3 maximum
Melting point	12–14°C
Added antioxidant	Vitamin E
Free fatty acids (%)	<1
Triacylglycerol content of oil	>97%
Fatty acid composition	**Rel. % (w/w)**
14:0	1–1.5
16:0	22–25
16:1(n-9)	0.5–1.5
18:0	5–8
18:1(n-9)	38–41
18:2(n-6)	10–12
18:3(n-6)	15–19
18:3(n-3)	0.2

[a]As produced by J. & E. Sturge, Selby, North Yorkshire, UK under the trade name Oil of Javanicus.

Extraction of Arachidonic Acid SCO from Mortierella alpina

The application of arachidonic acid (AA) rich oil for infant nutrition has led to the development of several commercial production processes over the past 15 years, all using *Mortierella alpina* (4,5) (see Chapter 5). *M. alpina* was selected because its level of AA can exceed 50% total fatty acids (TFA). Moreover, as an oleaginous fungus, it can accumulate high levels of triacylglycerol (TAG) lipids, and it is considered safe (6). AA is found both in the polar lipids and in the TAG. The TAG fraction is currently used as the commercial product.

For a number of years, this oil has been produced in full-scale fermentation and downstream-processing facilities. Companies active in commercial production include Suntory (Japan), Martek (U.S.), and DSM (Delft, The Netherlands); the literature also suggests developments in China (Wuhan Alking Bioengineering Co. Ltd) (7) and possibly South Korea. The DSM process, which is outlined here, produces an oil that is incorporated into infant formulas in conjunction with a compatible source of DHA. This incorporation has now been approved by many regulatory authorities, including the U.S. Food and Drug Administration (see Chapter 11). For details concerning the properties of the production organism, strain selection, and the fatty acid (FA) composition of the oils, the reader is referred to Chapter 5.

Process Design

A schematic overview of process steps that are common to most oleaginous microorganisms used for SCO production is given in Figure 13.1.

Fermentation. Starting from a working cell bank (either as spores or as vegetative mycelia), shake-flasks and inoculum fermentors are used for the initial phases of biomass production. Lipid production is not a goal in these stages; rather it is the generation of sufficient biomass of the desired macroscopic morphology (8). As for the main fermentation, TAG—and this includes the AA oil—are generally best produced under nitrogen limitation (1,9). The specific conditions for the fermentation process are discussed in Chapter 5. In summary, efficient production conditions can be maintained by providing a limited feed of the N source, and a nonlimiting, but controlled feed of the C source.

It is usually advisable to let the concentration of the C source drop to a low level by the end of the fermentation, since this is an expensive raw material. Any excess would be wasted during down-stream processing and may even have undesirable effects, such as color formation, as well as increasing the biochemical oxygen demand of the final effluent leaving the production plant. The organism is capable of consuming the lipid that it has laid down during the production phase. This phenomenon has sometimes been exploited because it is associated with an increase in the AA percentage in the lipid fraction (10) though, of course, the amount of lipid is now diminished.

Biomass Stabilization. Self-consumption of the lipid, however, becomes a problem when it occurs after the fermentation has been stopped, potentially affecting the quantity and quality of the product. Two relevant metabolic processes may occur at this point: (1) lipase activity, that lowers the TAG content of the oil; and (2) the subsequent catabolism of the FA. To prevent these processes from occurring, the biomass should be inactivated as quickly as possible once the production phase has ended. To investigate this, the wet biomass from a pilot plant fermentation was stored for 24 h at either −50, 4, 25, or 63°C. The samples stored at 4 and 25°C were tested with and without prior pasteurization at 63°C..No changes were observed at −50 and 63°C, showing that biomass is stable at temperatures that are too low or too high for metabolic activity. The data for the samples kept at 4 and 25°C are shown in Figure 13.2. It appears that prior pasteurization at 63°C was required and sufficient to stabilize the oil and FA levels of the biomass when kept at these intermediate temperatures.

In the DSM process, the biomass is dried by extrusion drying. This yields low-dusting granules with a narrow size distribution that are quite suitable for subsequent extraction. The dry, inactivated biomass is a rather stable production intermediate, but cool storage under N_2 is necessary for optimal quality, because it is susceptible to oxidative damage. When kept for several weeks at room temperature in air, the levels of unsaturated FA decrease. This also applies to samples stored for analysis.

Extraction, Refining, and Final Product. The dry biomass particles are extracted with hexane (5,10). This gives a crude oil that is quite stable. At this stage, many hexane-soluble cell components are still present, and it is commonly held that this includes endogenous antioxidants. These components are greatly reduced in the subsequent refining, bleaching, and deodorization steps. Therefore, the refined oil is protected by

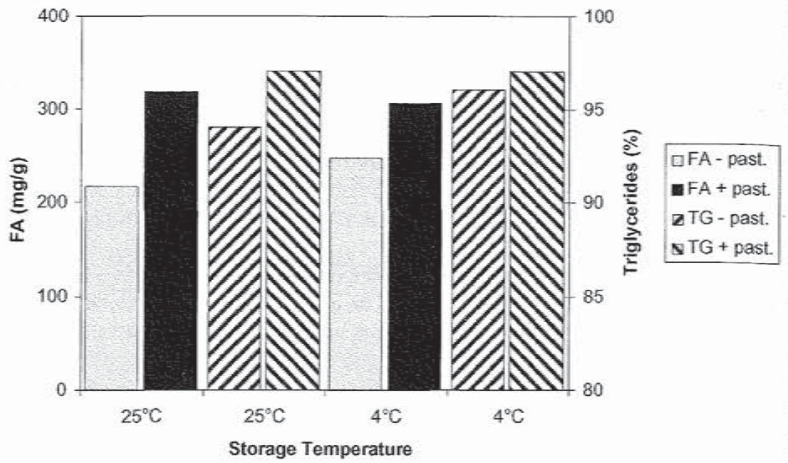


Fig. 13.2. Pasteurization of wet biomass of *Mortierella alpina* for oil stabilization. Solid bars: fatty acid (FA) content of dry biomass (mg/g); striped bars: triacylglycerols (TAG) in hexane extract (%w/w). Past., pasteurization at 63°C.

an antioxidant system, usually mixed natural tocopherols and ascorbyl palmitate. These are standard steps for high-quality edible oils; the only thing that is more or less specific for AA is the relatively small production scale for this speciality oil, and the oxidative susceptibility of the LC-PUFA therein.

The commercial product is a clear yellow TAG oil, with specified limits for unsaponifiables and FFA (Table 13.2). The final AA content is adjusted to about 40% TFA by adding vegetable oil. This is the oil that is used in conjunction with a compatible source of DHA to provide LC-PUFA in infant formulas.

Extraction of DHA-Rich Oils from Crypthecodinium cohnii *and* Schizochytrium *sp.*

Fermentation and Harvesting. Crypthecodinium cohnii and *Schizochytrium* sp. are two microorganisms used for the commercial production of PUFA by Martek Biosciences. Details of the two processes are provided in Chapters 6 and 3, respectively.

Schizochytrium sp. is a heterotrophic microalga belonging to the order Thraustochytriales within the phylum Heterokonta that can yield about 40% w/w DHA from its TFA production. *C. cohnii* is a unique heterotrophic marine dinoflagellate since DHA is almost the only PUFA present in its lipid and can be as high as 65% TFA (11). Table 13.3 shows a typical FA composition of both microorganisms as produced by fermentation, i.e., prior to processing and blending.

DHA is contained entirely within the cells and is distributed in both structural lipids (e.g., phospholipids) and storage lipid. The latter consists of TAG and is heat

TABLE 13.2
Specifications of the Commercial Arachidonic Acid (AA) SCO Obtained from *Mortierella alpina* by the DSM Process

General composition	
AA (g/kg oil)	min. 350
AA (%)	min. 38
Peroxide value (meq/kg oil)	<5
Unsaponifiable matter (% m/m)	max. 3
Free fatty acids (%)	<0.4
Appearance	Clear yellow liquid
Fatty acid composition	Rel % TFA
14:0	1–3
16:0	12–18
18:0	10–14
18:1	10–14
18:2	5–8
18:3	2–5
20:0	0.5–2
20:3	2–5
20:4	35–43
22:0	0.2–4
24:0	1–4
Stabilization	
Mixed Natural Tocopherols	250–500 ppm
Ascorbyl Palmitate	250 ppm

labile; consequently it requires due diligence during the down-stream harvesting of the cells and extraction of the oil. The viscosity of the broth may increase drastically due to production of extracellular polysaccharides (12). In addition, the pH tends to change rapidly during the final holding time. All these factors tend to increase the difficulties of product recovery. To ensure good recovery of the oil at optimum quality, speed of operation is an overriding factor because of the sensitive nature of the product to contamination, cell lysis, and PUFA oxidation. The processing equipment must therefore be of the correct type and size to ensure that the harvest broth can be processed within a satisfactory time limit. Nevertheless, this time limit may be extended by stabilizing the broth using heat or preservatives. Generally, the broth should be concentrated using centrifugation or ultrafiltration in order to make it compatible with the subsequent drying operation and to reduce the drying energy cost.

Drying is required to produce a stable biomass form that can be stored for an extended time without any microbial, chemical, or sensory deterioration. Biomass normally has a limit in the time and temperature to which it can be exposed without creating unacceptable decomposition of the oil. The choice is thus between long drying times at lower temperatures or brief exposure to more severe conditions. The normal choice is to use spray- or flash-dryers that have short exposure times. Spray dryers can handle large volumes and are suited for the size of fermentors used for

TABLE 13.3
Fatty Acid Composition of Oils Produced by *Crypthecodinium cohnii* and *Schizochytrium* sp. Prior to Final Processing and Blending

Fatty Acyl Group	*C. cohnii*	*Schizochytrium*
10:0	0–0.2	—
12:0	3–5	0–0.5
14:0	14–16	9–15
16:0	10–14	24–28
16:1	2–3	0.2–0.5
18:0	0–0.3	0.5–0.7
18:1	9–10	—
18:3(n-3)	—	—
20:3(n-6)	—	0–0.5
20:4(n-3)	—	0.5–1
22:5(n-6)	—	11–14
22:6(n-3)	50–60	35–40

microalgae. It is important to minimize cell lysis prior to drying in order to preserve the quality of the oil and minimize stickiness to the dryer. Drying conditions should be tailored for a heat-sensitive material to ensure that there is minimum loss of product, potency, nutritional value, or flavor deterioration. This should take into consideration the concentration of solids in the broth being harvested, temperature, air humidity, final moisture, nozzle design, and feed pressure. Both dewatering and drying can have a significant impact on extraction performance and product quality. Target moisture for dried biomass is usually between 4 and 6%.

It must be remembered that fermentation and product recovery are integral parts of an overall process. Because of the interaction between the two, neither stage should be developed or modified independently, since this may result in problems and unnecessary expense.

Pre-Treatment and Cell Disruption

Both microorganisms considered in this section are protected by extremely tough cell walls. In order to release their cellular contents, a number of methods for cell disintegration have been developed. These methods fall into three major categories: chemical, biological, and physical. Some of the methods have severe limitations with regard to large-scale application, compatibility with the product, or cost.

Knowledge of cell wall structure and composition is, therefore, important to optimize chemical methods and achieve cell lysis without damage to the DHA oils. For mechanical methods, size, shape, and degree of cross-linking of structural polymers are important factors to determine the ease of disruption. Nevertheless, mechanical methods, especially wet milling in high-speed agitator bead mills and high-pressure homogenizers, have demonstrated good performance on a large scale for cell disruption of microorganisms with tough cell walls, including microalgae. It is desirable to achieve as complete cell disruption as possible through the optimization of processing

variables including flow-rate, pressure, temperature, and disruption chamber design and operation. Some of the variables involved in cell disruption have been reviewed (13). The disintegration process, therefore, will strongly influence the solid-liquid separation in the downstream processing and the overall extraction yield.

The ease of cell disruption is also related to fermentation growth conditions. Fast growth rates, in general, produce cells having weaker cell walls since they do not have time to produce material to reinforce the cell wall structures. *Schizochytrium* sp., a faster growing algae than *C. cohnii*, possesses an intrinsically weaker cell wall and, as a consequence, energy requirements for its disruption are significantly lower.

The findings with these two marine organisms are in keeping with work with other microorganisms. For example, yeast cells that are in the exponential phase of growth are more susceptible to disruption than cells that are in the stationary phase (14); fast growing bacteria possess weaker cell walls than slowly growing ones and are easier to disintegrate by impingement (15). During the starvation or limited growth conditions that are used to promote lipid accumulation in oleaginous microorganisms, physiological signals may trigger the cells to reinforce the cell wall to prepare for survival (16). Similar phenomena have also been observed with micro-algae. The mechanical stability of micro-algae is therefore not a constant but depends on the strain being used, growth conditions, and the history of the biomass. In conclusion, biology and upstream variables have a major impact on downstream processing cost, speed, and efficiency.

Pre-treatment of broth or dried biomass is not necessary but could be useful in special situations, such as long-term storage or shipping for further processing. Although the main objective for pre-treatment is usually stabilization, pre-treatment methods and conditions could also improve extractability by weakening the cell wall. The most common pre-treatment is subjecting the broth to heat at pasteurization conditions. Great care should be taken, however, in order to minimize potential degradation of PUFA or other chemical reactions that would impart an off-flavor and make downstream processing more problematic.

Extraction and Refining

Historically, the three most common processes for recovering oil from plant seeds are hydraulic pressing, expeller pressing, and solvent extraction. Solvent extraction originated as a batch process in Europe in 1870. The modern solvent-based process usually consists of extraction by successive countercurrent washes with hexane of the previously cracked, flaked, ground, or pressed oleaginous material. The extracted meal is conveyed to a solvent recovery system, usually a desolventizer/toaster. The hexane is removed from the oil using evaporators and reused in the process.

The same process could be applied to SCO microorganisms with some modifications using hexane as a solvent. The main differences are in the pretreatment of the cells and in the disruption method used, as described previously. Cell disruption generates rather a wide distribution of cell debris particle sizes that need to be removed. Adjustments in equipment design and operating conditions are necessary in order to

process streams that are different in physical and chemical characteristics than those generated by oilseeds. Figure 13.3 illustrates the main unit operations used to extract SCO. The many advantages of this approach include cost, efficiency, and quality.

Once the oil has been obtained, a miscella winterization process is required to remove high melting point TAG (i.e., those having the most saturated fatty acyl residues) and other impurities. This is necessary when a clear oil at room temperature is required. It is also preferable for it to remain clear when stored in the refrigerator if the oil is being used in capsules; a cloudy oil can be seen in the capsules and may be off-putting to a customer that does not appreciate the physical chemistry of oils.

Lipid extraction from intact cells using solvents has limited success and is generally limited to laboratory practice. Different solvents were tested to extract lipids and other products from intact microorganisms. For instance, methanol/benzene mixtures have been used to extract lipids from yeast (14), but such procedures have very limited applications for the large-scale recovery of SCO. Supercritical extraction is another alternative but needs further development to make it attractive in terms of processing cost and extraction yield. This topic is covered in detail in Chapter 14.

DHA crude oil is unfit for consumption because of impurities, odor, and taste as well as a lack of clarity. Therefore, it needs to be refined. This is achieved using standard vegetable oil refining steps including degumming, caustic refining, bleaching, and deodorization. Impurities and minor components that are removed or reduced in the refining steps include FFA, water, phospholipids, minerals, carotenoids, sterols, tocopherols/ tocotrienols, waxes, and residual cell debris. Since the oil is sensitive to oxidation (up to 60% DHA prior to blending, Table 13.3), process conditions and speed of operation are more critical than when processing vegetable oils and have been optimized at Martek Biosciences Corp. for odor, taste, and oxidative stability. The deodorized oil is blended with high oleic sunflower oil, to standardize the DHA

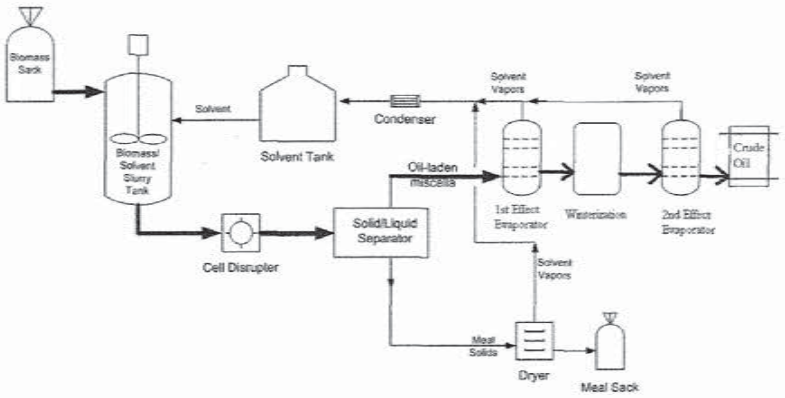


Fig. 13.3. Extraction flow diagram for recovery of SCO from *Crypthecodinium cohnii*.

concentration, and then it is stabilized by adding antioxidants, mainly ascorbyl palmitate and tocopherols.

The cost of extracting and refining SCO is, however, much higher that vegetable oils; vegetable oil processing has benefitted from economy of scale and decades of operational optimizing and fine-tuning. In addition, SCO requires a higher degree of good manufacturing practices that is closer to pharmaceutical standards than to those used by the food industry; it also requires a very stringent control of oxidation that limits the performance of the equipment being optimized.

Quality Aspects

Unlike crude oils from oilseeds and fish, the crude oils from *C. cohnii* and *Schizochytrium* sp. produced by fermentation are free from pesticides, aflatoxins, organophospho-insecticides, organochloro-insecticides, heavy metals, and other pollutants often found in fish oils such as polychlorinated biphenyls (17). This simplifies the final refining procedures aimed at removing impurities that have been co-extracted from the cells along with the TAG and therefore does not compromise the quality of the final oil.

Typical analysis of refined, bleached, and deodorized oil after blending with high-oleic sunflower oil is given in Table 13.4. A rigorous quality control is used at Martek Biosciences Corp. to ensure consistency and quality. At each step of the operation, exposure to heat, air, light, and heavy metals is minimized. State-of-the-art analytical work and a trained sensory panel are used to improve and maintain the highest quality standards for food and therapeutic applications.

The SCO from microalgae possess a remarkable oxidative and flavor stability. Figure 13.4 illustrates typical shelf-life stability of the *Schizochytrium* sp. oil under frozen conditions where only a minor change was detected over 2 yr. Further information regarding the safety aspects of these and other SCO is covered in Chapter 11.

Extraction of Oils from Microalgae

General Considerations

Phototrophic algae have been studied regarding their potential to produce several very long-chain polyunsaturated fatty acids (VLC-PUFA), predominantly EPA and AA. The major producers of EPA are the diatom, *Phaeodactylum tricornutum* (18,19); the eustigmatophytes, *Nannochloropsis* sp. (20,21), and *Monodus subterraneus* (22); and the red alga, *Porphyridium cruentum* (23). The latter was, until recently, the only algal source of AA (24).

The major impediment that prevents the use of microalgae as a source of VLC-PUFA is the relatively high cost of producing their biomass. Consequently, not much effort has been dedicated to developing down-stream processes of algal SCO in comparison to alternative sources. Novel panel photobioreactors, currently being tested, are expected to significantly reduce the cost of production.

TABLE 13.4
Typical Analysis of DHASCO™ Produced by *C. cohnii*[a]

Analysis	Typical Result
Free fatty acids (%)	0.03–0.1
Peroxide value (meq/kg)	0–0.5
Anisidine value	2–8
Unsaponifiable matter (%)	1–2
Moisture and volatiles (%)	0–0.02
Insoluble impurities	n.d.[b]
trans fatty acid	n.d.[b]
Heavy metals	n.d.[b]
Tocotrienols (ppm)	400–500
Fatty acid composition	**Rel. % (w/w)**
10:0	0–0.5
12:0	2–5
14:0	10–15
16:0	10–14
16:1	1–3
18:0	0–2
18:1	10–30
22:6(n-3)	40–45

[a]Final specification of the *C. cohnii* oil from Table 13.3.
[b]Not detected, n.d.

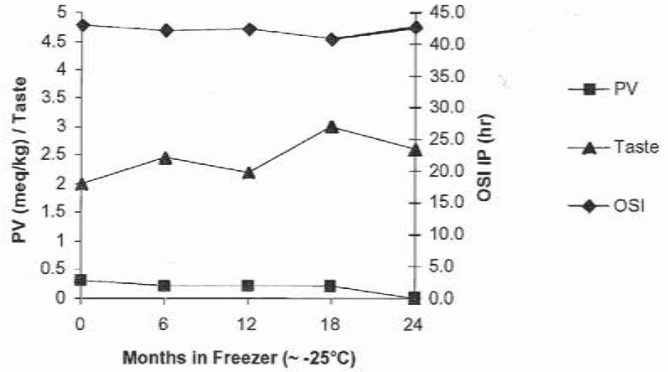


Fig. 13.4. Typical shelf-life stability of SCO derived from *Schizochytrium* sp. PV, peroxide value; OSI, oxygen stability index; IP, induction period.

Under stress conditions, such as nitrogen starvation, many microalgae can be induced to accumulate large amounts of oil. However, the accumulated TAG are mostly constructed of saturated and monounsaturated with little, if any, PUFA. When present, PUFA are predominantly located in the polar membranal lipids (25).

Unfortunately, the content of membrane lipids and, consequently, their FA components, are inherently limited. One of the limitations of fish oils as a source of a single PUFA is the co-occurrence of several PUFA in the oil; this requires expensive HPLC separations. The same applies to algal oils. Preparative HPLC, however, is the single most expensive component in producing the final product, affecting the cost of the purified product much more than the cost of producing the oil extract itself (26).

Separation of different lipid classes from each other is, however, much simpler. Thus, the search for a promising PUFA-rich algae should take into consideration not only the absolute content of the PUFA of interest, but also whether or not it is concentrated in specific lipid classes and to what extent other PUFA are present in these lipids. One possible approach is searching for algae with PUFA-rich TAG. Indeed, Cohen *et al.* (27) have hypothesized that some algae living in habitats characterized by rapid changes in environmental conditions can swiftly adapt by mobilizing LC-PUFA from their TAG to chloroplastic lipids. Based on this hypothesis they have isolated a microalga that was identified as the chlorophyte *Parietochloris incisa* (28). This alga was found to be the richest plant source of AA. While the alga can withstand very low temperatures, its optimal growth temperature is 25°C. Under nitrogen starvation, the proportion of AA was close to 60% TFA and the AA content over 20% dry wt., over 90% of AA was deposited in TAG (29). However, the down-stream processing of AA from this alga has not yet been studied.

Extraction

The recovery and separation of algal PUFA were recently studied in depth by Molina Grima *et al.* (19). This section will thus only briefly relate to aspects covered elsewhere in this review. Direct extraction of wet biomass *P. tricornutum* with 96% ethanol produced almost as much lipid (90%) as that from freeze-dried biomass (96%) (26). However, the cell walls of many species that contain potentially valuable oils are impermeable, requiring a cell disruption step, such as *P. incisa* and the astaxanthin-rich *Haematococcus pluvialis*. Bead mills have been successfully used to disrupt cells of *Scenedesmus obliquus* (30). The classical extraction method of plant lipids utilizes mixtures of chloroform, methanol, and water. However, these solvents are too toxic to be used for nutritional or pharmaceutical purposes. In most algal species, EPA is mostly found in polar lipids, mainly galactolipids. Unfortunately, the solubility of these lipid classes in most relevant solvents is not very high and results in high extraction volumes and incomplete recovery. However, since the final purification of FA requires their release from the host lipid, a simultaneous saponification/extraction process was shown to be advantageous with respect to recovery. Biocompatible systems, such as ethanol (96%) and hexane/ethanol (2:5 v/v), have been successfully used to extract FA from the lyophilized biomass of *P. tricornutum* following saponification (31). Similar purities were obtained using this process to separate EPA from *Isochrysis galbana* (32) and *P. cruentum* (33). The yields, however, were significantly lower reaching 43 and 25%, respectively.

Purification

Reverse-phase chromatography separates FA according to their chain length and the degree of unsaturation (34). However, this technique could be prohibitively expensive on a large scale. Preliminary separation of the PUFA-rich galactolpid fraction followed by urea treatment can reduce the cost by increasing the output and subsequently reducing the cost per run. Cohen and Cohen (35) demonstrated the potential of this method to purify EPA from the galactolipids of *Porphyridium* by successive elution of the trans-methylated oil with acetonitrile-water mixtures on C_{18} Sep-Pak filters. Over 80% of the EPA was eluted in three fractions with an EPA content that ranged from 85.4 to 93.2%. Yet, when reverse-phase chromatography was preceded by urea treatment, 85% of the EPA was recovered at 97% purity. Application of these methods resulted in an EPA concentrate of 97% purity. Similar methods resulted in an AA concentrate of 80% purity.

M. subterraneus is one of the leading algal sources of EPA. Over 70% of cellular EPA is concentrated in the galactolipid monogalactosyldiacylglycerol (MGDG), where it makes up 46% TFA. As mentioned previously, the difference in polarity between lipid classes allows for a much simpler separation that can serve to partially remove some of the other PUFA. Such separation could be accomplished by washing over a pad of silica gel. To simulate this method, lipid extracts of *M. subterraneus* were washed over silica gel cartridges with solvents of increasing polarities and the subsequent fractions were then analyzed (Cohen, Z., and Cohen, S., unpublished data). Transmethylation of the MGDG-rich fraction and urea fractionation of the resulting methyl esters resulted in EPA of 88% purity (Table 13.5). Even further improvements can be expected by incorporating several genetic modifications, *e.g.*, the galactolipids of *M. subterraneus* are made of two types of molecular species: the eukaryotic-like, with mostly 20:5 at both the *sn*-1 and *sn*-2 positions; and the prokaryotic-like lipids containing shorter (C_{14}-C_{18}) FA in the *sn*-1 position and 20:4 or 20:5 at the *sn*-2 position. These molecular species originate from two different biosynthetic

TABLE 13.5
Fractionation of the Lipids of *Monodus subterraneus* by Stepwise Elution from Silica Gel Cartridges, by Transmethylation, and Urea Fractionation[a]

	FA Composition (% TFA)										
Lipid fraction	14:0	16:0	16:1	16:3	18:0	18:1	18:2	18:3	20:3 n-6	20:4 n-6	20:5 n-3
Total lipid extract[b]	6	14	23	2	1	5	1	1	0.5	6	39
5% MeOH elution[c]	3	9	24	0.6	4	3	1	1	0.4	5	50
Urea fractionation				0.5			1	1	0.6	7	90

[a]The process was studied on a small scale only and was not optimized.
[b]Lipids were extracted by the Bligh and Dyer method (*Source:* Reference 37). TFA and eicosapentaenoic acid (EPA) content were 12 and 4.7% dry wt, respectively.
[c]Neutral lipids were washed away with chloroform. Galactolipids containing fraction were obtained by washing with 5% (v/v) methanol in water. Washes with higher concentration of methanol yielded other polar lipids with lower EPA content.

pathways (36). Any modification that will increase the share of the eukaryotic-like molecular species at the expense of the prokaryotic-like species would inherently also increase both the EPA content and its ease of purification.

PUFA such as AA, EPA, and DHA, are in current demand to treat various diseases and symptoms. However, inclusion of a PUFA as a drug component would require its purification to over 95%. Different sources would thus compete, not only in productivity, concentration, and cost of production, but also by the ease or difficulty to separate the PUFA of interest from other similar FA. When considering a source of PUFA, one should also consider in which lipids it is concentrated and what is its relative distribution with respect to other PUFA.

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Chapter 14

Supercritical Fluid Extraction of Lipids and Other Materials from Algae

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Introduction

The natural extracts and fine chemicals available on the market today are usually obtained by wet extraction processes based on volatile, flammable solvents like hexane, acetone, and halogenated hydrocarbons. Such solvents have a detrimental impact on the environment and constitute a health hazard; therefore they are subject to close continuous scrutiny and stringent regulations. Other drawbacks associated with these production processes are that they are energy intensive, create toxic disposal problems, pollute the atmosphere by the emission of volatile organic compounds, and pollute water bodies and soil through leaks and spills. Furthermore, wet extraction can never deliver extracts that are totally free of toxic solvents.

In recent years, supercritical fluid extraction (SCFE) has received increased attention as an important, environmentally compatible alternative to conventional separation methods. Supercritical fluids have good extracting power because their density can be controlled by changing the pressure or temperature. In addition, their low viscosity, high diffusivity, and low surface tension contribute to their extracting power by enhancing mass transfer inside a solid matrix. Supercritical CO_2, being nontoxic, nonflammable, inexpensive, and easily separated from the extracts, is the most commonly used extractant. An additional advantage of supercritical CO_2 is that its low critical temperature facilitates the extraction of thermolabile compounds without degradation.

SCFE is thus a clean extraction and separation process that can replace wet extraction in combination with the energy-intensive processes of distillation or evaporation. Being particularly effective for the isolation of materials of medium molecular weight and relatively low polarity, SCFE is particularly suitable for applications in the food, pharmaceutical, and cosmetics industries *inter alia*. In extraction applications, SCFE has already been used in such diverse areas as decaffeinating coffee, tertiary oil recovery, and separating petroleum heavy-ends.

Natural extracts are usually complex mixtures containing active compounds in different proportions. Some (like lycopene and β-carotene in algal extracts) may already be well-known for their nutritional and medicinal properties, whereas others that are present in small amounts have not been obtained in sufficient quantities for

their potential to be exploited. SCFE provides an easy fractionation procedure that can overcome this obstacle.

As discussed by Randolph (1), the combination of the ability to change the solubility power by varying the pressure and the favorable properties of supercritical fluids confers on them several special advantages for extraction processes, especially for the biotechnology industries. The high diffusivities exhibited by supercritical fluids reduce mass-transfer resistance and greatly enhance extraction rates, particularly for extraction from porous matrices (such as algal material) for which manipulation of external fluid mechanics has no effect on mass-transfer rates. Low surface tension facilitates penetrating and wetting the accessible regions with liquid solvents important to extract chemicals from cellular material. Solubility is extremely sensitive to changes in pressure and temperature; this permits the manipulation of the selectivity of an extraction. Supercritical fluids enable extraction of both solid and liquid materials; in the case of solid components, these can be crystallized within desired sizes and morphology, with obvious advantages for the pharmaceutical industry, in which product morphology can be critical to drug uptake rates or mechanical fragmentation procedures may be unacceptable due to thermal instability or contamination risks. SCFE can be performed at room temperature, facilitating separation and purification of thermolabile compounds. Extraction capacity for solutes can be very high, as illustrated by enhancement factors of 10^3 to 10^{12}. The restrictions and regulations as to the amounts of residual toxic solvents remaining after wet extraction methods obviously do not apply to extractions with supercritical fluids.

There are several main disadvantages of SCFE. The actual solubility of a compound is still much lower than those attainable in many wet extraction processes. Equipment costs are substantially higher, and the equipment expensive to operate. Complete information about the technology is not always available. Data on physical and thermodynamic variables may be inadequate: Optimization of SCFE is influenced by many unknown factors, such as matrix-solute interactions, porosity and shape of the matrix particles, moisture content, and amount of other substances that can also be extracted. Engineering information enabling proper design is lacking, and process modeling is far from satisfactory. Selectivity based on solute functionality can be achieved by the use of co-solvents, but this may obviate the advantage of minimal solvent residues in the final product (1).

Stahl *et al.* (2) have tested CO_2 supercritical extraction of many pure substances from several types of natural products (polyarenes, phenols, aromatic carboxylic acids, pyrones, and lipids) that differ in relative molecular weights, in the number and nature of their functional groups, and in polarity. Their findings indicate that at pressures up to 400 bar extractability falls sharply as the number of carbon atoms increases and as polar functional groups are added to the molecule being extracted.

Supercritical extractants may be characterized by the following rules of thumb. Hydrocarbons and other typically lipophilic organic compounds of relatively low polarity can be extracted in the lower pressure range, 70–100 bar. Introduction of strongly polar functional groups, such as –OH and –COOH, makes the extraction

116

more difficult. More strongly polar substances, (sugars and amino acids), cannot be extracted below 400 bar. When there are large differences in the commencement of boiling or sublimation and/or substantial differences in the polarity of the substances (dielectric constant), it is possible to obtain fractional separation by applying a pressure gradient.

Despite the many potential advantages offered by supercritical extraction, current applications are limited principally to certain families of natural products—lipids [fatty acids (FA) and triacylglycerols (TAG)], terpenes, and alkaloids. Lipids, being hydrophobic, are particularly suitable for supercritical extraction. Supercritical fluids are also used to extract ω-3 polyunsaturated FA, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Supercritical extraction of essential oils is currently a particularly active area of research (3).

Industrial supercritical extraction processes are well known: the technique is used both on a large scale, to decaffeinate coffee and extract hops, and on a smaller scale to extract high-value natural products, such as perfumes. According to Bartle *et al.* (4), over 200 patents for supercritical extraction processes have been registered in the U.S. alone, and as large a number outside the U.S.

In recent years, there has been increasing interest in using supercritical extraction as a method to separate high-value biochemicals from algae. This chapter presents an overview of the recent studies on the application of supercritical extraction in algae. It comprises the following sections: CO_2 as the preferred supercritical fluid, fundamental thermodynamic and kinetic aspects of supercritical extraction with CO_2, extraction of algae, and conclusions.

Carbon Dioxide as the Preferred Supercritical Fluid

It is the physicochemical properties of supercritical fluids that make them unique. The order of magnitude comparison presented in Table 14.1 indicates that while supercritical fluids have densities comparable to those of liquids, their viscosities and diffusivities are intermediate between those of liquids and those of gases. Thus, supercritical fluids have the solvent power of liquids with better mass-transfer characteristics than typical liquid solvents, and consequently separation efficiencies for supercritical solvent extractions can be appreciably higher than for liquid solvent extractions (5).

TABLE 14.1
Order of Magnitude Comparison of Density, Viscosity and Diffusion Coefficient of a Typical Gas, Liquid, and Supercritical Fluid (SCF)

Property	Gas	SCF[a]	Liquid
Density (kg/m^3)	1	700	10^3
Viscosity (Ns/m^2)	10^{-5}	10^{-4}	10^{-3}
Diffusion coefficient (m^2/s)	10^{-5}	10^{-8}	10^{-9}

[a]At $T_r = 1$ and $P_r = 2$.

TABLE 14.2
Temperature, Pressure, and Density of Fluids Used as Supercritical Solvents

Fluid	T_c (K)	P_c (bar)	ρ_c (kg/m^3)
Xenon	289.5	57.6	1,100
Trifluoromethane	302.0	46.9	516
Carbon dioxide	304.2	72.8	448
Ethane	305.4	48.2	200
Nitrous oxide	309.6	71.5	457
$CHClF_2$ (Freon 22)	369.2	49.1	310
Propane	369.8	41.9	870
Ammonia	405.6	111.3	240
Methanol	512.6	79.9	270
Water	647.3	217.6	320

Among the many gases that have been proposed as supercritical solvents, those most often used for SCFE are carbon dioxide, nitrous oxide, ethylene, and some Freons, the most attractive of which is carbon dioxide (Table 14.2). The solvent power of the previously mentioned fluids facilitates the extraction of compounds of different polarities and different molecular weights (M.W.). In practice, only a few common solvents satisfy all the necessary conditions for successful extraction, because of unfavorable mass-transfer and thermodynamic properties, high cost, and toxicity. In the supercritical fluid region, a pure component is highly compressible. Since solvent power is directly related to supercritical solvent density, the solvent power of a supercritical fluid can be varied continuously from liquid-like densities, at which the fluid is a good solvent, to low densities at which the fluid is a poor one.

The phase diagram of CO_2 is given in Figure 14.1. The physical and chemical properties of CO_2 make it the most used fluid for supercritical extraction. Its low critical temperature (304.2 K) makes it most suitable to extract thermolabile compounds. It is non-flammable, relatively inert towards reactive compounds, can be obtained easily at very high purity, at a very reasonable price, and is nontoxic (with a threshold limit value for airborne concentration at 298 K of 5000 ppm, rendering it less toxic than many other organic solvents). It is naturally abundant, and it is a gas at ambient temperatures. The latter property facilitates its easy recovery from the sample. The main disadvantage of CO_2 is that, being a non-polar compound, it has a low solvent power (3). At liquid-like densities, CO_2 has a viscosity of as little as 10% that of water and its surface tension is much lower than that of conventional organic solvents, hence the diffusivity of solutes in CO_2 is expected to be considerably higher. Accordingly, it may be expected that CO_2 will wet and penetrate complex geometries better than simpler liquids and therefore that solutes dissolved in CO_2 will diffuse faster within catalyst pores than the same solutes dissolved in conventional liquid solvents (6). The high critical and vapor pressures of supercritical CO_2 mean that CO_2-based processes are not only more expensive than those using conventional solvents but that they also require specialized equipment.

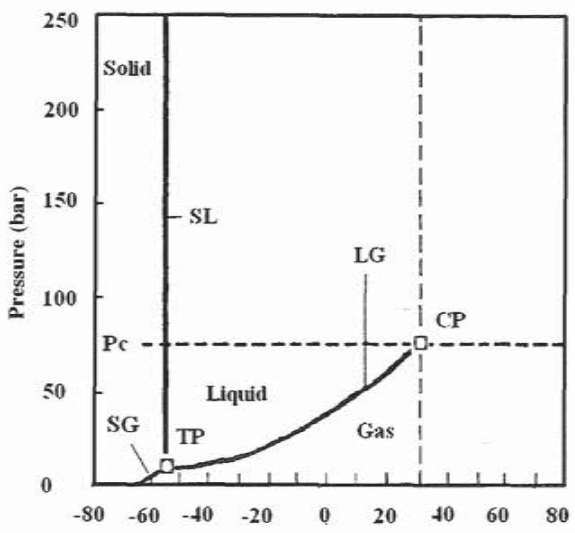


Fig. 14.1. Phase diagram of carbon dioxide. Abbreviations: SL, solid-liquid line; LG, liquid-gas line; SG, solid-gas line; TP, solid-liquid-gas triple point; CP, critical point.

Despite the advantages of supercritical fluids, particularly CO_2, very often their solvent power is insufficient, and a co-solvent (known as a polar modifier) is required. Among the substances most frequently used for this purpose, one can mention methanol, ethanol, acetonitrile, water, and dichloromethane in concentrations that may vary from 1 to 30%. Addition of a co-solvent decreases the diffusion coefficient, but this inconvenience is largely compensated for by improved solvent power. The choice of the modifier is guided by the nature of the solutes to be extracted, according to the principle of "similar dissolves similar" (3,7). Apart from the fact that modifiers improve the solubility of polar compounds, the effect of their addition under supercritical conditions is still unknown, as are the mechanisms by which they increase solubility (3,8). Although the addition of polar modifiers substantially improves the solvent power of a supercritical solvent, it also affects the selectivity, because the solubility of undesirable solutes is also improved.

Fundamental Principles: Thermodynamic and Kinetic Aspects

For the fundamental design of supercritical solvent extractors, in both fixed bed and countercurrent flow configurations, information is required on the solubility of the

solutes in the solvent phase, the equilibrium distribution between the matrix and the solvent phase, the rate of solute transfer from the matrix to the solvent, and the relationship between overall mass-transfer coefficients and operating variables (4,9–11). In general, there are two experimental procedures—static extraction and dynamic extraction—to study supercritical extraction; these two may also be combined to extract a given material (3):

In the static extraction mode, the cartridge containing the biomass is pressurized with the supercritical fluid to a given pressure and temperature, and then the flow of fluid is interrupted. After a predetermined period of time, the flow is re-established to permit recuperation of the extract. This method exploits the good diffusivity of supercritical fluids and enables the exact determination of the amount of co-solvent to be added. The method is usually applied to determine solubility and/or the influence of the co-solvent; its use in conjunction with particular analytical procedures is limited.

The dynamic extraction method differs from static extraction in that the flow of the solvent through the sample is continuous; this permits a faster and more complete extraction. An additional advantage of the dynamic method is that it can be coupled to an analytical procedure. The disadvantages are the large volumes of solvent required and the reduction in selectivity vis à vis the static method, resulting in a less pure sample.

The development of accurate methods to correlate and predict the solubility of solids in supercritical solvents is critical to designing supercritical extraction processes properly. This task is difficult because the behavior of fluids and their solutions in the supercritical region is not well understood. Several factors contribute to the complexity of the problem. The phase behavior of highly compressible pure fluids and their solutions, particularly multicomponent solutions, is far from understood. Current understanding suggests that the high density leads to solvent condensation or clustering about the solute, even in non-polar gases. There are large differences in size, polarity, and shape between the solvent and the solute. Standard solvents have relatively low M.W. The M.W. of the solute may be several orders of magnitude higher than the supercritical fluid, making the application of equations of state and parameter mixing rules a complicated task.

Much effort has been invested in measuring the solubility of substances in supercritical fluids and developing models for their behavior. Real processes, however, usually involve mixtures, and the solute phase may be different from that of the pure substance, i.e., it may be a liquid rather than solid, or it may be adsorbed in a substrate. Under these circumstances, the solubility only gives an indication of the relative extractability of the substances as a function of temperature and pressure. However, it is still an important parameter in designing the correct operating conditions to obtain an extract of desired composition (4).

Many attempts have been made to relate solubility directly to pressure and temperature using Hildebrand's solubility parameter, δ (12); standard thermodynamic principles (the fugacity of a given component is identical in each phase it appears) (5,13), coupled with equations of state for the supercritical fluid phase (14,15); and

empirical models. Unfortunately, none of the relationships developed so far is able to describe the solubility of different compounds in supercritical gases over a wide range of pressures and temperatures; in many cases the estimation of the constants in these equations is very difficult or impossible (16). The interested reader is directed to the specialized literature for further details (13–16).

A typical procedure for estimating the increased solubility resulting from the real nature of the gas is performed through an enhancement factor E, as follows:

$$y_2 = \frac{P^s_2}{P} E \qquad [1]$$

$$E = \frac{\varphi^s_2}{\hat{\varphi}^s_2} \exp\left[\frac{v^s_2(P - P^s_2)}{RT}\right] \qquad [2]$$

where y_2 is the mole fraction of the solute in the gas phase, P^s_2, and P are the vapor pressure of the solid and the total pressure (at temperature T) respectively, v^s_2 is the molar volume of the solid (assumed to be independent of temperature), R is gas constant, φ^s_2 and $\hat{\varphi}^s_2$ are the fugacity coefficients of the pure solid and the dissolved solid, respectively. The latter two unknowns can be calculated using an equation of state, as described by Sandler (14) and Orbey (15). In fluids such as CO_2, the E values fall within a range of only 1.5 orders of magnitude for substances with a variety of polar functional groups (excluding strong bases such as ammonia). The enhancement factor provides a measure of the extent to which the pressure enhances the solubility of the solid in the gas: $E \rightarrow I$ as $\rightarrow P^s_2$. Enhancement factors of 10^4 are common, and values over 10^{10} are known, namely, for squalene in supercritical CO_2 (17).

In reviewing the solubility data available for a very large number of compounds and the many equations that have been proposed to correlate solubility directly with density, Bartle *et al.* (4) reached the conclusion that the best equations are:

$$\ln(y_i \rho) = a' + b'\rho \qquad [3]$$

$$\ln \frac{y_i P}{P_{ref}} = A' + B'\rho \qquad [4]$$

where y_i is the mole fraction of the solute in SF, ρ is the density of the SF and P_{ref} is a reference pressure (usually taken as 1 bar), a', b', A' and B' are empirical constant parameters.

In addition to solubility data, information on the mass-transfer characteristics of the supercritical extraction process is required. The extraction process from natural materials such as plants, beans, seeds, or algae, may involve the release of solutes from porous or cellular matrices into a solvent *via* a mass-transfer process. The solutes fixed or trapped in a matrix by physical or chemical forces must be released

and transferred to the supercritical fluid by dissolution or desorption. The dissolved solutes then diffuse out of the matrix to the surface of the particle. Finally, they move across a stagnant film around the particle to the bulk fluid phase. Because the solubility of many compounds is low in supercritical fluids and because mass transfer is generally rapid, extraction processes may be equilibrium limited rather than mass-transfer limited (1).

A general picture of the extraction of soluble species from solid matrices includes four different mechanisms. If there is no interaction between the solute and the solid phase, the process involves simple dissolution of the solute in a suitable solvent that does not dissolve the solid matrix. If there are interactions between the solid and the solute, then the extraction rate of solute desorption in the presence of the solvent and the adsorption isotherm may have significant influence on the equilibrium state. Most solid extraction processes, such as activated carbon regeneration (18) and catalyst regeneration (19), fall into this category. A third mechanism involves swelling the solid phase or destruction of the solid texture by the solvent, accompanied by extraction of the entrapped solute through one of the first two mechanisms (such as extraction of essential oils). The fourth mechanism is reactive extraction (similar to reactive distillation), in which the insoluble solute reacts with the solvent, and the reaction products are soluble and hence extractable (such as the extraction of lignin from wood). Extraction is always followed by another separation process in which the extracted solute is separated from the solvent (20).

Supercritical extraction has been extensively tested experimentally to extract from solid matrices and much quantitative information is available for many raw materials. Nevertheless, very little is known about the mechanism of the process. The rate of mass transfer, the axial dispersion, and the diffusion into the pores are engineering parameters needed to design the extraction system. Neither data on these parameters nor reliable prediction techniques is currently available; understanding of the actual mechanisms by which the solute is retained by the solid matrix is incomplete. In addition, extraction is usually a multicomponent process, and many of the required thermodynamic properties for the components are unknown (20,21).

Typical experiments concerning the extraction of biochemicals from uncrushed algae show that the extraction rate is very low and almost independent of the supercritical fluid conditions. These experiments indicate that the extraction rate is controlled by the mass-transfer resistance in the algal cell walls. To overcome this obstacle, it is necessary to disrupt the cell wall; this is usually done by one of the common techniques for wall destruction, such as grinding.

A typical experimental curve for SCFE from algae with disrupted walls at constant extraction conditions shows two stages, as has been found for oil extraction from crushed soybeans (22). In the initial stage, the solute concentration is high and the extraction rate is constant; this indicates that the internal resistance of the solute in the matrix is negligible and that the extraction rate is controlled mainly by the convectional mass-transfer resistance in the supercritical fluid. As the solute concentration decreases, the effect of solute diffusion in the matrix increases, and the extraction rate

119

decreases with time. From a practical point of view, the maximal percentage extraction recovery is limited by the interaction between the solute and the matrix.

Cygnarowicz-Provost *et al.* (23) developed the following empirical correlation to represent the experimental overall volumetric mass-transfer coefficient A_pK for the two stages:

$$A_pK = A_pK_0 \exp\left[\ln C\,(X_0 - X)/(X_0 - X_{shift})\right] \quad [5]$$

where A_pK_0 is the initial mass-transfer coefficient, X is the solute concentration in the matrix at time t, and X_0 and X_{shift} are at the initial time and the time at which the diffusion mass transfer in the matrix starts to control the extraction, respectively. Parameter C has a value between 0 and 1 and is determined from the relationship between the overall volumetric mass-transfer coefficient A_pK at $X = X_{shift}$ and its value at the initial time A_pK_0.

The extraction rate at any time is directly proportional to the concentration driving force in the supercritical fluid and is given by the following equation:

$$R = A_pK\,(Y^* - Y) \quad [6]$$

where R is extraction rate, and Y and Y^* are the concentrations of the solute in the supercritical fluid and at equilibrium under the extraction conditions (pressure, temperature, etc.).

Extraction of Biochemicals from Algae

It is a well-documented fact that microalgal biomass constitutes a reservoir of potentially valuable natural compounds. According to Kobayashi *et al.* (24) some species of microalgae have developed metabolic pathways leading to the accumulation of high quantities of biochemicals, such as carotenoids (to act as antioxidants under conditions of light stress), saccharose or proline (to protect the cells against hypersalinity), exocellular polysaccharides (to enable the cells to survive desiccation), and unsaturated FA (to induce changes in membrane viscosity and hence to provide protection against temperature variations and salinity). The main products currently being commercialized are carotenoids, phycobilins, FA, polysaccharides, vitamins, sterols, and other biologically active compounds. The most valuable fine chemical derived from algae is the carotenoid β-carotene. Carotenoids are the most widely distributed class of pigments in nature and have essential biological functions in animals (25). Some species of algae (*Euglena*, *Haematococcus*, and *Chlorella*) also produce other carotenoids, such as astaxanthin and canthaxanthin, in significant quantities.

In general, extraction and purification of algal products are processes similar to those used for plant cells, yeasts, and bacteria, that is, wet extraction using liquid organic solvents. The increased demand for natural products processed without any chemical contact has motivated the development of new extraction methods. In

microalgae, supercritical extraction has been used to obtain lipids, FA such as EPA, and carotenoids such as β-carotene. These compounds are sensitive to heat, light, and oxygen and can be easily degraded.

The solubility of carotenoids in supercritical CO_2 depends on the length of the hydrocarbon chain and the presence of functional groups as well as on the entrainment effect of other lipids (fatty oils, TAG, and waxes) (2,26). The solubility of β-carotene in CO_2 has been reported in several papers (27–29). Similarly, a number of studies have focused on supercritical extraction with CO_2 from plants and algae (2,30) and on supercritical extraction of other carotenoids, such as astaxanthin and its esters, from Antarctic krill (31) and bixin from annato seeds (26,32,33). The authors will describe in some detail the information available on the supercritical extraction of valuable biochemicals from some of the most important species of microalgae.

Chlorella vulgaris

Chlorella microalgae are characterized by high growth rates, relatively easy handling, and high biological value. Mendes *et al.* have published several studies on the feasibility of supercritical extraction of carotenoids from *C. vulgaris* in a flow apparatus (26,34). In their first work (34), the algal cells were first crushed and then extracted with highly pure CO_2 in a temperature range of 298.15 to 353.15 K and pressures of up to 40 MPa. The extraction yields at pressures approaching 35 MPa were similar to those obtained with liquid hexane and acetone at atmospheric pressure (34). Later, Mendes and coworkers (26) extended their experiments to include supercritical CO_2 extraction of whole and crushed algae at 313.15 and 328.15 K and pressures up to 35 MPa. Lipid extraction yield from whole cells increased either with increased pressure at constant temperature or with increased temperature at constant pressure. The extraction curves consisted of two zones, one controlled by the solubility of the outer lipids of the cells and the other, by diffusion within the cells. Extraction yields of carotenoids at 328.15 K increased greatly with increased pressure and concentration of carotenoids in the extracts. The weight of the carotenoid fractions also increased over the course of the extraction. Visual inspection of the residues after supercritical extraction showed that their color changed according to the degree of extraction attained: they were yellower and less red when the extraction was more intense. The increased viscosity of the extracts was due to a reduction in the concentration of FA and an increase in the wax fraction (at the plateau zone of the extraction curves). The extraction yield of canthaxanthin (the major carotenoid component in both the whole cells and the supercritical fluid extracts) also increased as a function of the CO_2 volume at 35 MPa and 328.15 K (26). Crushing the algal cells before the extraction process resulted in substantially higher yields. The yield also increased with increased pressure, and at 20 MPa it decreased with temperature, but the effect was less pronounced. The maximum yield of lipids (based on dry weight of crushed algae) obtained by supercritical extraction was 13.3% mass at 35 MPa and 328.15 K, vs. 5% mass for whole cells. However, the yields for wet extraction with acetone and hex-ane were higher, being 16.8 and 18.5% mass, respectively.

Mendes *et al.* (26) drew the following conclusions from their study on supercritical extraction of algae. Higher pressures lead to more efficient supercritical extraction of lipids and carotenoids from crushed microalga cells. A small increase in temperature at higher pressures gives a slightly higher extraction yield. At lower pressures, the temperature has an opposite effect on the extraction yields of carotenoids and other lipids. Supercritical extraction of intact algal cells results in lower yields of carotenoids and lipids (5% dry wt at 35 MPa and 328.15 K), while the use of disrupted cells gives higher yields (13.3% mass under the same conditions). At 35 MPa, the yield of carotenoids from supercritical extraction of crushed cells compares favorably with yields from acetone and hexane extraction. Varying both the extraction pressure and the volume of CO_2 facilitates modification of the carotenoid fraction in the extracted oils. Addition of vegetable oils to the crushed cells as entrainers should be considered as a possible alternative means to increase the extraction yield and protect the carotenoids from degradation.

In their third study, Mendes et al. (35) attempted to correlate all their experimental results using a nonsteady state model similar to the following models: that of Lee *et al.* (36) to extract canola oil with supercritical CO_2; that of Cygnarowicz-Provost *et al.* (23) to extract lipids from fungi (*Saprolegnia parasitica*) with CO_2 and CO_2-10% ethanol; that of Schaeffer *et al.* (37) to extract the monocrotaline alkaloid with CO_2 and CO_2-5% ethanol from the seeds of *Crotalaria spectabilis*; and that of Rao and Mukhopadhdyay (38) to extract spices with CO_2 from cumin seeds. Examination of the experimental results indicated that a constant overall mass-transfer coefficient was unable to describe the entire extraction curve because at a certain point, the controlling rate-determining step switched from external mass transfer to intraparticle diffusion, with the corresponding reduction of the extraction rate. The variation in the overall mass-transfer coefficient was taken into account using the empirical correlation developed by Cygnarowicz-Provost *et al.* (23) and Mendes *et al.* (35).

For lipid extraction of a slightly crushed (milling time =15 s) biomass of *C. vulgaris* (lipid content 50% dry wt) at 328.15 K and 200 and 350 bar, the shift from the diffusion-controlled regime occurred when 22% of the total lipids had been extracted. Microscopic observation showed that under this treatment the cell walls were disrupted—but not completely destroyed—and that there were no lipids outside the cell walls. For completely crushed (milling time = 45 s) cells, lipids colored with carotenoids appeared outside the cells. The shift from the diffusion-controlled regime occurred when 55% of the lipids had been extracted, a value similar to that found by Cygnarowicz-Provost *et al.* (23) for the extraction of lipids from fungi. The overall mass-transfer coefficient, A_pK_0, was found to increase with pressure; for instance, at 200 bar (328.15 K) it was 0.6 kg/m^{3}s, while for 350 bar (328.15 K) it was 2.2 kg/m^{3}s. The model provided a good representation of the extraction of the lipids from the algae (35).

Botryococcus braunii

The unicellular green alga *B. braunii* (race A) produces and accumulates hydrocarbons (C_3–C_{25} dienes with an odd number of carbon atoms and a triene, $C_{29}H_{54}$). About 95% of these hydrocarbons are localized outside the cell wall but are not bound to it. These compounds can be used as substitutes for paraffinic and natural waxes in the cosmetic and pharmaceutical industries (39).

Mendes *et al.* (39) studied the supercritical extraction with CO_2 of the slightly crushed microalga in a flow apparatus at 313.15 K and pressures between 12.5 and 30 MPa. Under these conditions, both the extraction yield and the hydrocarbon fraction in the extracts increased with pressure, and at 30 MPa the yield rapidly approached total extraction of the lipids. The hydrocarbon fraction in the extracted lipids was also greater at higher pressures because of the localization of the hydrocarbons on the outside of the algal cell wall. No chlorophyll or phospholipids were detected in the extracts (39).

Experimental results indicate that the supercritical extraction of *B. braunii* with CO_2 is an efficient method to extract both lipids and hydrocarbons and that at higher pressures, the time for CO_2 extraction may be shorter than that for hexane extraction due to the substantial increase in solubility of these compounds with pressure. An additional advantage is that supercritical CO_2 extracts only the hydrocarbons and not chlorophyll, whereas hexane extracts both types of compounds. Analysis of the extraction curves showed that during the period of the fast initial rates there is a selective effect of pressure. At higher pressures, the dissolution rate of the external hydrocarbons was higher than that of the intracellular lipids; this results in increased hydrocarbon fractions in the extracts. The amounts of C_{27} and C_{29} hydrocarbons extracted decreased proportionally during the progress of the extraction, while the proportion of C_{31} hydrocarbons increased. The lower–M.W. compounds were initially extracted preferentially, due to higher solubility in supercritical CO_2 (39).

Dunaliella

The alga *Dunaliella* is one of the richest natural sources of β-carotene, and *Dunaliella* is currently grown and harvested on a commercial basis. The spray-dried alga is marketed as a health food and vitamin supplement. When grown under optimal conditions of high light intensity, high salinity, nitrate and phosphorous deficiency, and extreme temperatures, these algae can accumulate up to 8% dry wt β-carotene.

Gamlieli-Bornshtein (40) studied the possibility of using fractionation schemes to purify β-carotene from *Dunaliella* by supercritical extraction with CO_2 in a continuous flow extractor. A comparison of the solubility of the 9Z and all-E isomers of β-carotene obtained from *D. bardawil* in supercritical CO_2 at 44.8 MPa and 313.15 K showed that the 9Z isomer was almost four times more soluble than the all-E isomer (1.92×10^{-5} vs. 7.64×10^{-5} g /g CO_2) (29). When supercritical extraction was applied to a carotenoid concentrate obtained from *D. bardawil* (29% w/w β-carotene) or a freeze-dried powder of the algae (3.1% w/w β-carotene), a selective separation of the 9Z/all-*E* isomers of carotene was obtained. A 39% recovery of β-carotene with 80% purity of the 9Z isomer was achieved at the initial stages of extraction (40 mL CO_2). The extraction rate for β-carotene from the freeze-dried algal powder was slower than that from the carotenoid concentrate, and resulted in a reduction in the recovery and

121

purity of the 9Z isomer. This finding indicated that the internal mass-transfer resistance was significant even at the initial extraction rate. Isomer purity and recovery could be enhanced by grinding the algae power.

Haematococcus pluvialis

The green microalga *H. pluvialis* has been widely studied as a source of natural astaxanthin. The levels of astaxanthin that accumulate in this alga depend on the growth and culture control methods. Typical levels range from 0.5 to 2% dry wt (41). However, when this microalga is in cyst form, the very hard cell wall makes it difficult to extract the astaxanthin, and the bioavailability of astaxanthin in intact encysted cells is low (39). Mechanical disruption of the cells is usually required to enable quantitative extraction of astaxanthin, although hot dimethyl sulfoxide would probably also be successful, as was found for extraction of astaxanthin from the yeast *Phaffia rhodozyma* (41). Kobayashi *et al.* (24) found that astaxanthin was not released from intact cyst cells upon extraction with 90% (v/v) acetone for 1 h; consequently they compared several procedures to improve astaxanthin extractability from cyst cells. Astaxanthin extractability from lyophilized or enzyme-treated intact and heat-acetone treated cyst cells was either negligible or not observed following treatment with the following commercially available lytic enzymes: Actinase E, chitinase, Fangase I, Funcelase, Novozym 234, pectinase, protease, Protease K, Tunicase R70, Uskizyme, Yatalase, and Zymolyase 100T. However, Kitalase, Cellulase Onozuka-RS, and abalone acetone powder enhanced extractability (24). In addition, heat acetone-treated (chlorophyll-free) cyst cells treated with a mixture of the latter three enzymes led to a threefold increase in extractability, compared to intact cells. Treatment with 40% (v/v) acetone for 2 min at 353.15 K, followed by lyophilization or exposure of the cells to specific lytic enzymes, enabled extraction of 70% of the astaxanthin present in *H. pluvialis* cells.

Mendes-Pinto *et al.* (39) tried other approaches to disrupt encysted cells of *H. pluvialis* and facilitate astaxanthin recovery. For this purpose, they submitted the biomass to the following processes: autoclave 30 min, 394.15 K, 1 atm; HCl 0.1 M, 15 min and 30 min; NaOH 0.1 M, 15 and 30 min; enzymatic treatment with a mixture of 0.1% protease K and 0.5% driselase in a 0.2 M phosphate buffer, pH of 5.8, at 303.15 K for 1 h; spray drying, inlet 453.15 K, outlet 388.15 K; and mechanical disruption with a cell homogenizer. The best results were obtained with mechanical disruption processing and enzymatically treating the biomass. These results were supported by SEM studies that showed the effect of the different treatments on the cell wall (39).

Valderrama *et al.* (42) studied the influence of operating variables on the supercritical CO_2 gas extraction of *H. pluvialis* and *Spirulina maxima*. Three extraction runs were performed with *H. pluvialis*. In run 1, the samples were crushed by cutting mills prior to extraction. In run 2, the samples were crushed by cutting mills and then manually grinding with dry ice prior to extraction. In both cases, extraction was performed with pure supercritical CO_2. A third run was performed using samples treated as in run 2 but extracted with supercritical CO_2 and ethanol (9.4% mass) as a co-solvent.

Two extraction runs were performed with *S. maxima*. In run 1, the samples were first crushed by cutting mills and then extracted with pure CO_2. In run 2, the samples were treated as in run 1 but extracted with supercritical CO_2 and ethanol (10%) as a co-solvent. Since the blue pigment phycocyanin is not soluble in CO_2, soluble materials that are primarily composed of lipid-soluble compounds were extracted, while the phycocyanin remained with the residue inside the extractor vessel.

The extraction profiles of astaxanthin at 333.15 K and 300 bar with pure CO_2 and with CO_2 + ethanol gave an extract yield for run 1 (slightly crushed cells) lower than 1% mass. For run 2 the samples were ground twice; the yield increased significantly, reaching close to 1.3% mass. These findings illustrate the importance of breaking the cellular wall on extraction efficiency. The addition of ethanol as a co-solvent increased the yield slightly to about 1.6% mass. The maximum total recovery of astaxanthin, calculated from its initial and residual content in the alga (0.0147 and 0.0004, respectively), exceeded 97%. For phycocyanin extraction, the addition of a co-solvent (10% w/w ethanol) had a strong effect on the extraction yield of lipid substances. The total extraction yield of about 3% mass corresponded well to the average lipid content of 3.3% in *S. maxima* reported in the literature (42).

P. rhodozyma

The red yeast *P. rhodozyma* is characterized by the synthesis of carotenoid pigments (25). Lim *et al.* (43) studied the extraction of astaxanthin from *P. rhodozyma* containing about 1,000 ppm of the active component. Before extraction, the yeast was disrupted in a bead mill and spray dried at 371.15 K, and then supercritical extraction was performed using pure CO_2 as the supercritical fluid and ethanol as the modifier. Astaxanthin was extracted from the treated cells under various temperatures (313.15, 333.15, and 353.15 K) and pressures (102 to 500 bar) (36,43). Extraction yields of total carotenoids and astaxanthin at constant temperature increased as the pressure was increased up to 200 bar at the three temperature levels studied. A substantial increase was observed for a pressure of 200 bar. The extraction yields of carotenoids and astaxanthin increased upon increasing the temperature for a given CO_2 density, a finding that can be explained by the increase of vapor pressure of carotenoids and astaxanthin with temperature. The highest yields of carotenoids and astaxanthin obtained were 84 and 90%, respectively (50 g CO_2), at 313.15 K and 500 bar, when the density of CO_2 was about 1.0 g/cm^3. The effects of CO_2 flow rate on the astaxanthin extraction yield were determined at 333.15 K and various pressures levels (400, 500, and 600 bar) with CO_2 superficial velocities of 0.27 and 0.54 cm/min. Increasing the CO_2 flow rate resulted in a slight increase in the total astaxanthin yield, but to a lesser extent than that of other components such as total lipids and total carotenoids. The extraction yields of astaxanthin and carotenoids were approximately doubled during the initial 30 min of extraction by increasing the flow rates from 0.27 to 0.54 cm/min (36,43).

Addition of ethanol as a CO_2 modifier at 1, 5, 10, or 15% (v/v), increased the total yield of astaxanthin by about 24% at 333.15 K and 500 bars, but only by 9% at 313.15 K. The extraction rate with the modifier increased by about 65% (vs. extrac-

tion with pure CO_2) when 14 g CO_2 at 313.15 K was made to flow through the extractor, and increased by about 50% when 30 g CO_2 at 333.15 K was used. These findings may be explained by the fact that the addition of a modifier increases the solubility of CO_2 and causes swelling of the matrix, thereby increasing the internal volume and the surface area for the contact of the material with the supercritical fluid (36,43).

The astaxanthin concentration obtained by one-step supercritical extraction with CO_2 at a constant pressure and temperature was similar to that obtained by acetone extraction. Lim *et al.* (43) used a two-step pressure gradient (at 313.15 and 333.15 K) to concentrate the astaxanthin in the extract. Since large amounts of lipids were obtained in the first extraction, a more concentrated astaxanthin product was obtained in the second extraction step at 313.15 and 333.15 K. With this two-step pressure gradient operation, it was found that the concentration of astaxanthin in the extract increased by about 4-fold at 313.15 K and 10-fold at 333.15 K, but its yield decreased to about 40–50%. Carotenoid concentrations were enhanced by about 3.6-fold at 313.15 K and 13-fold at 333.15 K, when compared with acetone extraction (36,43).

According to Lim *et al.* (43), for supercritical extraction runs at the same temperature, higher pressures gave higher yields of carotenoids and astaxanthin from the disrupted yeast due to the increased solvent power of CO_2. The extraction yields of carotenoids and astaxanthin increased gradually up to 200 bar at 313.15, 333.15, and 353.15 K; a substantial increase was observed when the pressure exceeded 200 bar. At 313.15 K and 500 bar, the highest yields of carotenoids and astaxanthin with equal amounts of CO_2 (50 g) reached 84 and 90%, respectively. At the same CO_2 density, the extraction yield of astaxanthin increased with increasing temperature due to the increase in vapor pressure of astaxanthin. Addition of 15% (v/v) ethanol as a co-solvent at 500 bars at two temperatures, 313.15 and 333.15 K, increased the extraction rate by about 50 and 65% at the beginning of the extraction and increased the total yield of astaxanthin by 9 and 24%, respectively. A two-step gradient operation (changing the pressure from 300 to 500 bar at 313.15 and 333.15 K) increased the concentration of astaxanthin in the second fraction at 500 bar by about 4-fold and 10-fold at 313.15 and 333.15 K, respectively. Total carotenoid concentration was increased by about 3.6-fold at 313.15 K and 13-fold at 333.15 K, in comparison with acetone extraction (36,43).

Mortierella alpina

Arachidonic acid (AA) is a long-chain polyunsaturated fatty acid of the n-6 family. Many *Mortierella* species produce substantial quantities of AA (over 50% of total fatty acids) and/or EPA, depending on the species and culture conditions. In addition, *M. alpina* is an oleaginous fungus, that is able to accumulate high levels of triacylglycerols, and is considered safe (44). An extract containing 73.5% (w/w) AA was prepared from the recovered mycelium. AA-rich oil has been produced in full-scale fermentation and downstream-processing facilities using wet extraction. The oil is incorporated into infant formulas in conjunction with a compatible source of DHA (44).

Parietochloris incisa

The freshwater green alga *P. incisa* is being extensively investigated as a source to produce AA (45). Extraction of AA from *P. incisa* by supercritical CO_2 is currently studied in the authors' laboratory (Korin, E., Wisniak, J., and Cohen, Z., unpublished results). The results obtained so far indicate that the extraction efficiency is very low due to the resistance of the cell wall. To improve extraction efficiency, several conventional methods for rupturing the cell wall, such as mechanical grinding, ultrasonic cavitation, osmotic shock, and enzymatic reaction, have been tested. Preliminary experiments carried out with 1 g algal biomass under supercritical extraction conditions with CO_2 (313 K, 27.2 MPa, flow rate 1 mL/min, and 1 h extraction time) indicated that mechanical grinding of the biomass for 10 min, increased the extraction efficiency of AA to 12.6%, compared with 2% for the alga without treatment. Increasing the extraction time of the crushed algae to 1.5 or 2 h increased the extraction efficiency to 20.5 and 23.0%, respectively.

Conclusions

SCFE is a relatively new method with great promise to extract high-value biochemicals from algae. Compared with conventional extraction liquids, supercritical fluids exhibit good transport properties, such as high diffusivity, low viscosity, and low surface tension, that improve the mass-transfer processes inside the matrix. The fact that the density of a supercritical fluid changes drastically near the critical point facilitates control of solubility in the supercritical fluid by manipulation of the temperature and the pressure of the extraction process. Supercritical CO_2 is the most frequently used solvent because it is nontoxic, non-flammable, and inexpensive, and it can be easily separated from the extracts. Furthermore, it is environmentally friendly and its low critical temperature facilitates the extraction of thermolabile compounds without degradation.

As in conventional liquid extraction, extraction rate enhancement requires disruption of the cell wall. The addition of modifiers, such as ethanol, increases the solubility of the solute and significantly improves the extraction rate and recovery of the desired extracted components. The supercritical fluid can also extract undesired components from algae, and it is therefore important to examine whether the product contains harmful biogenic components that might exist in the algae.

SCFE is a relatively expensive procedure, and therefore its application as an alternative to wet extraction of algae can only be considered for the extraction of high-value biochemicals, such as β-carotene from *Dunaliella*, astaxanthin from *Haematococcus*, and AA from *P. incisa*. In light of the fact that supercritical CO_2 is an environmentally friendly solvent, its use will become more and more attractive as concerns about environmental issues become more pressing.

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Chapter 15

The Future Development of Single Cell Oils

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Introduction

The potential of microbial systems to produce oils and fats on a large scale has been recognized for over 100 years, but only within the past ten years has this potential been transformed into a successful commercial process. During the intense political and economic turmoil of World Wars I and II, research and development in microbial oil production was driven by strategic defense issues. In peacetime, the strategic defense drives were replaced by the utilization of industrial wastes (e.g., whey from cheese production), or alternative, lower cost production systems for the equivalent of high-value oils produced by specialty crops (e.g., cocoa butter, and evening primrose oil substitutes). Although these incentives led to rapid development of the understanding of the capabilities of growing oleaginous microbes in deep tank culture, the improving efficiencies of agricultural production and global distribution dropped worldwide oilseed prices to levels at which microbial production was simply not economically competitive.

The recent commercial success of the specialty single cell oils (SCO) enriched in docosahexaenoic acid (DHA) and arachidonic acid (AA) can be attributed to three major factors: the accumulated knowledge base gained in earlier attempts to scale up other SCO processes; a critical need for specific characteristics in an oil by the infant formula industry; and no alternative plant or animal sources of oils with the required characteristics. As a result of the commercial success for the DHASCO® and ARASCO® process, the understanding of deep tank cultivation of oleaginous microorganisms, and the harvesting and processing of the oils contained therein, has expanded at an extremely rapid pace. However, the SCO process will again come under a strong competitive challenge if the needs of the infant formula industry change or if an alternative plant or animal source is identified that can fulfill the requirements of the infant formula industry. Furthermore, if new opportunities for SCO expansion are to be realized, new applications need to be identified that give the SCO process unique advantages in a manner similar to those of DHASCO and ARASCO in infant formulas.

A Short History of the Commercial Production of SCO

To better understand the future direction of SCO technology and application, it is important to learn the lessons of past successes and failures. The early development of

SCO production has been well reviewed by Ratledge [(1), see also Chapter 1] who noted that prior to the 1960s, interest in SCO was primarily based on academic curiosity tempered by strong prospects for commercial production should the need arise. In the last 30 years, full-scale production became a reality for several processes, but only in a few cases did this also become an economic reality and generate a truly sustainable commercial process. Although many SCO sources and applications were proposed in the past 30 years, only six have been scaled to commercial level.

Cocoa Butter Equivalent from Apiotrichum curvatum

Based on earlier work of Hammond (2), a team from the New Zealand Department of Scientific and Industrial Research led by Davies attempted to commercialize this yeast-based SCO process for cocoa butter equivalent (CBE) production in 1988 (3). The group successfully scaled the process to 250 m^3 and demonstrated a production process using oleagenous yeast that produced a palm oil equivalent. Although the production economics were based on the use of a discounted whey feedstock (a cheese processing by-product), the projected operating costs were still over \$700/T in 1988 and no commercial production of the SCO-CBE took place. At today's commodity price for soy oil \$230/T or CBE at \$2,000/T, the process is clearly not economically viable.

γ-Linolenic Acid Oil from Mortierella isabellina

Originally using *Mortierella isabellina* and later *Mortierella ramanniana*, the Idemizu Corporation (Japan) produced a SCO rich in γ-linolenic acid (GLA) for the domestic Japanese market in 1988 (4). Originally, the oil was used as a food additive, but this SCO source is no longer on the market. It is still unclear how much oil was produced or why it was taken off the market, but it is likely that it was still uncompetitive with the plant-based GLA oils such as evening primrose oil, borage oil, and blackcurrant seed oil.

GLA Oil from Mucor circinelloides

There is a much clearer picture of the development of a GLASCO from *Mucor circinelloides* (formerly *Mucor javanicus*) by Ratledge (5) and the ultimate scale-up by J. & E. Sturge Ltd (UK). Commercial production began in 1985 in 220 m^3 stirred tank fermentors that produced about two tons of oil per batch. Although the process was not closed down until the sale of the company in 1990, not many batches were produced since the market for a supplement or food additive containing GLA remained small and was well served by the plant-based sources of GLA.

DHA Oil from Crypthecodinium

In the late 1980s, Kyle's group at Martek Biosciences was also developing a series of SCO and quickly found an application for a unique oil (DHASCO) produced by the dinoflagellate algae *Crypthecodinium cohnii* (6). The application depended on a

requirement by the infant formula industry for an oil rich in DHA, but devoid of eicosapentanoic acid (EPA). These requirements could not be met by any known plant or fish oils and, as a result, commercial production of DHASCO in 200 m^3 stirred tank fermentors by Martek Biosciences Corp is still growing strongly today. A detailed account of this process is given in Chapter 6.

AA Oil from Mortierella

In the late 1980s, Kyle's group also developed an oil rich in AA for infant formula applications from the fungi *Mortierella alpina* (7). Like DHASCO, there is still no plant-based alternative, and animal sources (e.g., egg yolk) are more costly on an AA basis than ARASCO. Martek scaled up the process and then entered into an agreement with Gist-brocades (now DSM) for production; the product is now being produced in 200 m^3 fermentors (for further details and information please refer to Chapter 5).

DHA Oil from Schizochytrium

In the early 1990s, Kelco Corporation scaled up the production of a second DHA-rich SCO using the marine microbe, *Schizochytrium* sp. first described by Barclay (8). Although this process became very efficient and low cost, DHASCO was already selected by the infant formula industry, therefore the DHA oil from *Schizochytrium* was relegated to compete with fish oil (\$1,200/T) for a position in the food additive industry. It is still uncertain if this oil can compete with fish oil in food applications other than in the gel-cap nutraceutical market. An account of the development of this process and its current status is reviewed in Chapter 3.

The overall production of SCO in 2003 is estimated to be about 800 T, of which about 60% was ARASCO and 30% was DHASCO (Fig. 15.1). The expansion of this application is so rapid that the 2003 output alone is probably equivalent the total SCO output of the previous 30 years. However, the short-term production of GLA-rich oils in the 1980s and previous production of *Schizochytrium*-based DHA-oils (S-type) for the nutraceutical market changes the overall historical product mix slightly (Fig. 15.1).

Future Sources

Since there have been two successful examples of the commercialization of SCO, the hunt is on to find alternative sources of the same oils. These may come from classical screening of new microbes or the genetic modification of lower cost production systems (microbial or agriculture) to produce oils that are substantially equivalent to DHASCO and ARASCO.

New Sources of SCO

Since the discovery and application of the SCO from *C. cohnii*, many groups have searched for other dinoflagellates related to *C. cohnii* that could be used as an alternative source of DHASCO. Thus far, no alternatives have emerged from this group of

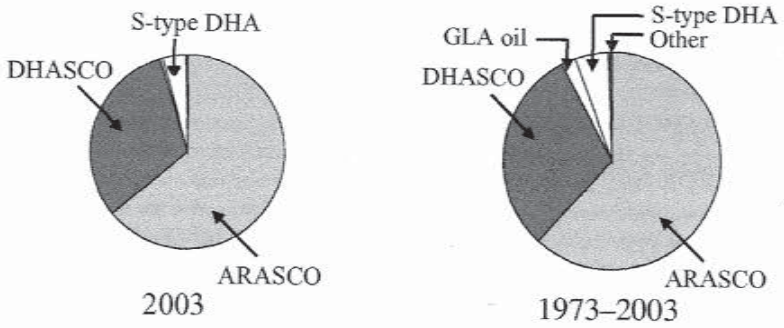


Fig. 15.1. Relative proportion of commercial single cell oil production in 2003, and from 1973–2003, attributed to various oil types: γ-linolenic acid (GLA) oil refers to oil from *Mucor circinelloides*; ARASCO is oil from *Mortierella alpina*; DHASCO is from *Crypthecodinium cohnii*; and docosahexaenoic acid (DHA) S-type is oil from *Schizochytrium* sp.

algae. Other algae have been identified as potential DHA and EPA producers, but these are generally photosynthetic and have not been scaled up to produce SCO (9). Search for chytrids other than *Schizochytrium*, however, has provided potential alternatives for that SCO. Phylogenetically similar organisms have been reported including *Ulkenia* (10) and *Labyrinthula* (11), but the oil composition of these strains appears to be virtually identical to that originally reported for *Schizochytrium*; this makes their discrimination very difficult. Because all the chytrids appear to have a relatively high content of n-6 docosapentaenoic acid (not to be confused with the n-3 docosapentaenoic acid precursor of DHA found in most fish oils), they are all easily distinguished from the DHASCO produced by *C. cohnii*.

Certain marine fungi represent possible candidates to produce AA-containing SCO. However, none have yet been reported to have the production efficiency of *M. alpina*, and most are producers of AA-rich phospholipids—not AA-rich triglycerides (12). Perhaps the closest alternative to the ARASCO product is an oil produced by the green alga *Perietochloris*. This alga produces over 50% of its weight in triacylglycerol and the fatty acid (FA) composition is almost identical to that of ARASCO (13,14).

Searches for new producers of SCO will continue; there is no doubt that new species and strains will be identified with unique characteristics that will permit the option of their use as a replacement for the existing SCO, or for use in completely new applications. Some of the recent developments that have taken place using mutant strains of *M. alpina* are detailed in the review given in Chapter 2.

Genetic Engineering of Microbes

The mutagenesis and selection of a yeast using classical techniques to produce an oil with a composition similar to cocoa butter (i.e., high stearic acid content) has already

been undertaken successfully (15). But, as discussed previously, the economics are still not favorable to produce a CBE from yeast. Yeasts grow very well in fermentors; if the genes for VLCPUFA biosynthesis can be transferred to an oleaginous yeast, the production costs for the more valuable SCO could be reduced significantly. Key regulator genes involved are those coding for the Δ5 and Δ6 desaturases and PUFA elongases. Attempts to isolate these genes from *Mortierella* have been successful; they have been transferred to yeast as a model system, to identify the genes rather than as a production system and yeast producing EPA and AA have now been reported (16–18).

An alternative approach involves identifying good potential SCO-producing photosynthetic algae and converting them into a heterotrophic production mode. It was originally believed that one characteristic that distinguished algae capable of growing in the dark in the presence of glucose (i.e., heterotrophically) from photoautotrophic algae (99% of all algal species) was the presence of an active glucose transporter. Apt and colleagues confirmed this supposition by converting the photoautotrophic algae *Phaeodactylum tricornutum* into a heterotroph by transforming the algae with a gene for glucose transport (19). This now opens up the potential of converting other photosynthetic alga into economic producers of SCO.

Genetic Engineering of Plants

Once genes coding for the various proteins involved in the biosysnthesis of EPA, AA, and DHA are isolated there is the possibility that they can also be transferred to agronomic plants for large-scale, low-cost production of DHASCO and ARASCO equivalents in field crops. If successful, this could eventually replace the SCO production technology for these products. However, the task may prove to be more daunting than originally anticipated. Initial attempts involved trying to transfer the genes from *Photobacterium profundum*, a DHA-producing bacteria isolated from the intestines of some deep water fish (20), but it was quickly realized that this organism did not produce DHA using the conventional FA biosynthetic pathway involving Δ5 and Δ6 desaturases and PUFA elongases as found in plants. Rather, DHA was produced using a unique polyketide-based pathway (20).

Another alternative source of genetic material was *Schizochytrium*, but once again the biosynthesis proved to involve a polyketide pathway even though this was a eukariotic organism (21). Isolating genes from the fungus *Mortierella* has been more productive, and these genes have been used to tranform yeast as described previously. The genes have also been used to convert Canola to produce EPA and AA (21). The biosynthetic pathway for DHA in mammals, however, involves additional desaturations and elongations of EPA to form 24:6; this is then cycled through one stage of β-oxidation to produce DHA (22). This "Sprecher Shunt" was proposed because of the inability to find a Δ4 desaturase and the general belief that such a desaturation cannot physically occur so close to the carboxyl end of the FA. Whether or not a Δ4 desaturase exists in any DHA-producing microbe is still hotly disputed.

Finally, even if a plant could be engineered to produce DHA (or AA), it may not be able to produce an oil as highly enriched (up to 50%) in the VLCPUFA as is found

in SCO without disrupting the biochemistry and physiology of the other plant tissues including the roots, leaves, stems, and flowers. As a result, this high level of enrichment may remain as a key differentiator between a SCO and a plant-based oil. Even if a plant could produce an oil with a substantial VLCPUFA enrichment, the oil would have to be harvested, processed, and stored without oxidation. Since this has proven to be very difficult even for flaxseed oil that contains high levels of 18:3(n-3), it may require the development of new transportation, extraction, and process technology for the new highly unsaturated plant oils. Oils that have a high propensity to oxidize, will generate heat on oxidation and may need to be treated with Ethoxyquin similar to fish meal (contains 5–10% fish) prior to transportation to prevent combustion. In addition to the requirement for a new process technology for the plant-based SCO equivalents, there is still a strong political and social reluctance to use genetically modified organisms (GMO) in foods. In the European Union, for example, no GMO materials are allowed in infant formulas. Thus, the production of the first plant producing AA or DHA may actually be the lowest hurdle to commercialize such a product.

Future Uses of SCO

It has now been well established that SCO can be produced in large quantities for commercial applications. From the experience of the past 30 years, however, it is also clear that the future commercial use of SCO will depend on their differentiation from existing or future alternatives, or the identification of specific market niches in which the higher cost of SCO is offset by the value of the new application.

Infant Formula

The use of DHASCO and ARASCO has been well established in the infant formula industry with nearly every major infant formula company worldwide using these oils for enrichment purposes. Due to the sensitivity of these products, it is unlikely that this will change in the next 5–10 yr. The high concentration of DHA and AA in these SCO minimizes their addition; public concern over the use of GMO products suggests that even if lower cost GMO plant sources were available, they would not be used in the foreseeable future. Thus, the production quantities of DHASCO and ARASCO should continue to rise as their market penetration increases, but they will likely level off at a production quantity of about 4,000 T/yr within 5 yr.

New Food Applications

The use of SCO in new food applications will depend on the application, commodity pricing of alternatives, and delivery form of the SCO. With respect to VLCPUFA, the primary competition will be fish oil. Fish oil has two major concerns: oxidative instability in food products due to the high levels of unsaturated FA that lead to organoleptic problems; and the potential for carrying contaminants such as dioxin, PCBs, methyl mercury, and other pollutants that concentrate in the fish oils. To overcome these problems fish oils must be purified and encapsulated to be usable in conventional food-based systems. There is less need for additional stabilization of SCO such as DHASCO that is highly enriched in only DHA, since it has no extraneous PUFA that may act as sites of oxidation. Thus, on a DHA for DHA basis, the price of the purified and encapsulated fish oil may be not too different from that of DHASCO.

Release of oils in the stomach, as is the case for fish-oil gel-caps or many microencapsulated fish oil products incorporated in certain food applications, results in premature oxidation of the oils as well as the potential for odiferous eructation (or "fishy burps"). To fully exploit the use of SCO (or fish oil) rich in VLCPUFA for food applications, it is still necessary to develop new delivery systems that allow gastric protection and postgastric release of the oils. Such delivery systems also need to allow the use of the encapsulated oils in a food matrix of high water activity (e.g., yogurt, spoonable dressings, or drinks) as well as in a matrix of low water activity (e.g., powdered formulations, food bars, breads). Such delivery systems will be developed in the future and this should greatly expand the use of SCO in mainstream food products. By 2010, such applications could increase SCO production by an additional 2,000 T/yr.

Animal Applications

When considering SCO applications, one should also consider the other parts of the microbial biomass and applications therefrom. The use of the whole SCO biomass, without the additional extraction costs and losses, may offer an economic opportunity for functional nutrition in the animal field. A number of patents have been filed on the use of SCO microbial biomass in animal feeds to improve the DHA content of the animal or a part thereof that is to be consumed. Examples include feeding of *Schizochytrium* biomass to chickens in order to produce high-DHA eggs (23), or feeding cows with a protected fish-oil product to produce DHA-enriched milk (24). Such concepts are limited by the extent of bioconversion of the feed DHA into the DHA content of the salable food product, without incurring substantial losses in general metabolism, conversion into other unused body parts, or losses in the feces. In competition with this approach is a cracked-egg product containing highly deodorized and purified fish oil at a much lower cost, and a milk product to which highly deodorized fish oil is added during the UHT (ultra-high temperature) packaging process at a price far lower than providing a DHA-enriched feed to the cow.

Although there may be niche markets to provide animals with SCO biomass containing certain FA to enrich food product from those animals, there may be a much larger market to provide the SCO biomass for the health benefit of the animal itself. Microbial sources of DHA, for example, are in the natural food chain of all aquatic animals. As the aquaculture industry grows and develops, the knowledge base of the nutritional requirements of many farmed fish species is also growing. In aquaculture, hatcheries and nurseries today are providing formulated feeds that represent the sole source of nutrition for these animals, much like infant formula for human babies. In this respect, the microbial DHA biomass represents the natural source of these materials for larval fish and crustaceans, or the equivalent of "mother's milk" for human

infants. Just as DHA and AA have been recognized as key components of a human infant's nutrition, they are now being recognized as key nutritional components in other animals. Consequently, there will be a rapidly growing market for the use of SCO microbial biomass in early stage animal nutrition, particularly in aquaculture.

Finally, many carnivorous aquatic animals, such as salmon and shrimp, have a high requirement for fish-meal and fish oil in their diet. It takes about 5 kg of wild caught fish to raise 1 kg of farmed salmon; this puts a serious concern on the sustainability of this industry. Furthermore, the toxin levels in farmed salmon were recently recognized to be about fivefold higher than wild caught salmon (25). It is now believed that these high toxin levels simply come from the bioaccumulation of toxins from the feed (salmon feed is about 40% fish meal and 10% fish oil). Both the lack of sustainability and the bioaccumulation of toxins argue for the use of an alternative to fish-meal and fish oil in this industry.

It has recently been established that the key component in the fish meal and fish oil that cannot be replaced by plant-sourced materials is DHA. A fish meal/oil alternative using SCO biomass has been proposed that will ensure the future sustainability and safety of farmed salmon. Such an environmentally responsible "Clean & Green" approach does not come without a cost, but a 15% premium on the price of end-products (like the premium on DHA/AA supplemented infant formula) will probably be accepted by most consumers. If this is correct, then a major expansion in the production and use of the SCO biomass may be the key to the future of the salmon-farming industry.

Conclusion

The commercial development of SCO has gone through a series of ups and downs since the first recognition of their potential over 100 yr ago. Initially SCO were academic curiosities. Then, there were several attempts at commercialization, but each failed due to lower cost alternatives on the market. The success story of DHASCO and ARASCO in infant formulas was based on being in the right place at the right time with no commercial alternative available for the industry. Since this industry is reluctant to change, it is unlikely that alternatives will replace DHASCO or ARASCO in the near future unless there is a substantial reason for the change. The scale-up and commercialization of these two oils however, has validated the concept proposed and encouraged by all the previous work on other SCO. Without the ground work and knowledge base built so extensively by previous SCO work, the commercial success of the DHASCO and ARASCO processes would never have been realized. It is with this background that one can look forward to a bright future for SCO and SCO biomass in solving many industry and socio-economic problems worldwide.

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Index

A

Acetic acid
  production of, by petrochemical industry, 107–108
  uses of, 109–116
Acetyl-CoA, carboxylation of, 140
Activated carbon regeneration, 227
Adults, single cell oils studies in, 190–192
Algae
  extraction of biochemicals from, 228–235
  supercritical fluid extraction of lipids and other materials from, 220–235
Alzheimer's disease, 185
Animals
  applications for single cell oils, 245–246
  single cell oils studies in, 186–188
Apiotrichum curvatum, cocoa butter equivalent from, 240
Aquaculture, 47, 245
Arachidonic acid (AA), 53, 73–81, 138
  abnormalities in metabolism in brain, 185
  biosynthesis of, in *Parietochloris incisa*, 64–66
  commercial demand for, 73–74
  commercial production of, 21
  conversion to eicosapentaenoic acid, 142
  effect of cell density on content, 63–64
  extraction of
  from *Mortierella*, 206–208, 234–235, 241
  from *Parietochloris incisa*, 235
  in glycerophospholipids of brain gray matter, 182
  induced accumulation of, in *Parietochloris incisa*, 61–63
  in membrane function, 185
  nutritional advantages of, 9
  presence of
    in body tissue, 183
    in human milk, 182
  role of, in *Parietochloris incisa*, 66–68
  sources of, 5, 74
Arachidonic acid-producing *Mortierella alpina*, 21–29
ARA-rich oils, 12–14
ARA-rich single cell oils, 9–10
ARASCO® process, 165, 166, 175, 176
  commercial success for, 239
  in infant formulas, 161, 168, 246
Aspergillus niger, production of citric acid from, 6
Astaxanthin, 124, 127–129
  Haematococcus pluvialis as source of, 127–129, 232–233
  Phaffia rhodozyma as source of, 129, 233–234

B

BASF, 16
Biochemicals, extraction of, from algae, 228–235
Bio-rational approach to technology development, 38–43
Biosynthesis
  of arachidonic acid in *Parietochloris incisa*, 64–66
  of eicosapentaenoic acid, 140
  of PUFA in *Porphyridium cruentum*, 55–56
Bipolar disorder, 185
Blakeslea trispora, production of, for β-carotene, 127
Borage oil, 204, 205
  as source of GLA, 6
Botryococcus braunii, extraction of biochemicals from, 230–231
British Nutritional Foundation (BNF), 88

C

Candida albicans, genetic engineering of, 131
Candida curvata (now *Cryptococcus curvatum*), 7

Canola oil, 2
Canthaxanthin, 124
Carbon dioxide as preferred supercritical fluid, 222–224
Carbon sources
  alternative, for heterotrophic production of docosahexaenoic acid by marine alga *Crypthecodinium cohnii*, 107–121
  in cell growth, 145
  ethanol as, 116–120
  lipid production from, 120–121
  uses of alternative, 108–109
β-Carotene, 124–127, 129, 228–229
  Blakeslea trispora as source of, 127
  Dunaliella salina as source of, 124–127, 231–232
  extraction of, 126–127
γ-Carotene, 129
Carotenogenesis, 128, 129
Carotenoids, 228–229
  extraction of, from *Chlorella vulgaris*, 229–230
  production of, using microorganisms, 124–131
Catalyst regeneration, 227
Cell density, effect of, on arachidonic acid content, 63–64
Cerebrosides, presence of, in brain, 182
Chill-sensitive mutants, selection of, in *Porphyridium cruentum*, 57–59
Chlorella vulgaris, extraction of carotenoids from, 229–230
Chlorella zofingiensis, 129
Chlorococcum sp., 129
Chloroplastic lipids, 55–56
  modification of, 59–60
Citric acid, production of, from *Aspergillus niger*, 6
Classical strain screening, 90–91
C/N ratio, 54–55, 80
  lipid accumulation and, 145
Cocoa butter equivalent, 7–8
  from *Apiotrichum curvatum*, 240
  from yeast mutants, 7
Concern levels in safety evaluation, 164
Continuous culture, 150
Corn oil, 2
Crypthecodinium cohnii, 100
  alternative carbon sources for heterotrophic production of docosahexaenoic acid by marine alga, 107–121
  docosahexaenoic acid from, 9, 151, 208–210, 240–241
  as production organism, 89–90
  single cell oils produced by, 165
Crypthecodinium oil, 10, 11
Cryptococcus curvatum, 7
Cultivation strategies for eicosapentaenoic acid production by microalgae, 149–150
Cultivation systems for microalgae, 147–149
Culture age, influence on eicosapentaenoic acid production, 144
Cyclooxygenases (COX-1, COX-2), 185
Cytochrome b_5, 28
Cytochrome P450 monoxygenases, 185

D

De novo synthesis, 59, 63
  of oleic acid (OA), 140
Δ5 Desaturase, 27
Δ6 Desaturase, 26–27
Δ12 Desaturase, 26
Δ5 Desaturase-defective mutants, 23
Δ6 Desaturase-defective mutants, 24–25
Δ9 Desaturase-defective mutants, 25
Δ12 Desaturase-defective mutants, 23–24
n-3 Desaturase-defective mutants, 25
DHA45 oil, 162, 165–166, 175
  safety of, 168
DHA-enriched eggs, 47
DHA-enriched milk, 47
DHAGold, 11
DHASCO® process, 165, 166, 175, 176
  characteristics of, 95–97
  commercial success for, 239
  industrial production of, 91–95
  in infant formulas, 161, 168, 246
  safety of, 97
DHASCO-S, 11, 12, 162, 165, 175, 176

DHASCO™, production of, 9
Diacylglyceryltrimethylhomoserine (DGTS) in *Parietochloris incisa*, 60–61
Dietary Supplement Health and Education Act (DSHEA) notification, 172
Dietary supplements, 172
Digalactosyldiacyglycerol (DGDG), 56
Dihomo-γ-linolenic acid (DGLA), 76
  commercial production of, 21
  production of, 13, 23
Dinoflagellates
  production of single cell oils by, 86–97
  characteristics of DHASCO™, 95–97
  Crypthecodinium cohnii as production organism, 89–90
  industrial, of DHASCO™, 91–95
  safety of DHASCO™, 97
  significance of docosahexaenoic acid in, 88–89
  strain selection and optimization, 90–91
  synthesis of docosahexaenoic acid by, 88–89
Dioxins in fishmeal, 12
Docosahexaenoic acid (DHA), 53, 138
  application of *Ulkenia*-derived, 105
  association with marine fish oil and, 86
  in body tissue, 183
  in brain development, 99, 183–185
  development of, using *Schizochytrium*, 36–48, 208–210, 241
  in glycerophospholipids of brain gray matter, 182
  heterotrophic production of, by marine alga *Crypthecodinium cohnii*, 107–121
  in human milk, 182
  importance of, 8–9
  microbial sources of, 12
  nutritional importance of, 9, 36–37
  production of, by marine microalgae, *Ulkenia* sp., 99–105
  in retinal development, 99
  significance of, in dinoflagellates, 88–89
Docosahexaenoic acid (DHA) oils, 10–12
from *Crypthecodinium cohnii*, 208–210, 240–241
  as nutritional supplement, 47
  properties of, when derived from *Ulkenia*, 101–102
  quest for, 8–9
  safety of the biomass and extracted, 46–47
Docosapentaenoic acid (DPA), 10, 101
Domoic acid, 43
Double mutants defective in both Δ12 and Δ5 desaturases, 24
Down-stream processing of single cell oils, 202–219
DSM, 10
Dunaliella, extraction of β-carotene in, 231–232
Dunaliella salina, 124
  production of, for β-Carotene, 124–127
DuPont, 16
Dynamic extraction method, 225

E

Ectoplasmic nets, production of, from thraustochytrids, 41–42
Eicosanoids, 36, 73, 138
Eicosapentaenoic acid (EPA)
  anti-inflammatory effects of, 13, 36–37
  biosynthesis of, 140
  commercial production of, 21
  importance of, 8–9
  Monodus subterraneus as source of, 216
  nutritional importance of, 36
  potential market size for, 13
  production of
    by microalgae, 13, 144–153
    by microorganisms, 150–153
  proportion of, in monogalactosyldiacylglycerol, 59
  sources of, 140, 142–143, 213
  structure and significance, 138–140
Eicosatrienoic acid, production of, 13, 24
Elongase, 27–28
Elongase-defective mutants, 25
Endomyces vernalis, 1
Environmental factors, influence on eicosapentaenoic acid production, 146–147

Enzyme genes, analysis of, involved in PUFA biosynthesis in *Mortierella alpina,* 26–28
Ethanol
as carbon source, 116–119
production of, by fermentation, 108
Eukaryotic lipids, 56
Europe, safety regulations in, 172–175
European Community Scientific Committee on Foods, 162
European Food Safety Authority (EFSA), 162, 173, 174–175
European Society of Pediatric Gastroenterology and Nutrition (ESPGAN), 88
European Union (EU)
responsibility of, for food regulation, 161
single cell oils approved in, 175
Evening primrose oil *(Oenothera biennsis),* 6, 204
GLA as component of, 5
Expeller pressing, 211

F

Fatty acids (FA)
accumulation of, following nitrogen starvation, 62
bacterial synthesis of, 142
role of, in brain function, 182
Fed-batch culture, 149–150
Fermentation
in developing large-scale, 4–5
of *Mortierella alpina,* 80–81
scale-up of technology, 43–46
Fischer-Tropsch synthesis process, 110
Fish oils
attempts to use as food ingredient, 36
investigation of dietary benefits of, 8–9
purification and encapsulation of, 244–245
as source of eicosapentaenoic acid, 140, 142
Food, Drug, and Cosmetic Act (FDCA), 162, 163
Food additives
defined, 169–170
safety of, 163
Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act (FDCA), 169
Food and Drug Administration (FDA)
responsibility of, for food regulation, 161
Toxicological Principles for Safety Assessment of Direct Food Additives and Color Additives Used in Food, 162
Food ingredients
regulatory requirements of, 169–171
safety evaluation of, 162–163
Foods
applications for single cell oils, 244–245
novel, 172, 173–174

G

Galactolipids, 65
molecular species composition of, in *Porphyridium cruentum,* 56–57
Gangliosides, presence of, in brain, 182
Gas chromatography, 5
Generally recognized as safe (GRAS) determination, 47, 161, 170, 171–172, 176
Genetically modified organisms
European Union resistance to using, 244
successful production of, 14–15
Genetically modified plant oils, competition from, and single cell oils, 14–17
Genetic engineering, 131
in improving microalgal strains, 153
of microbes, 242–243
of plants, 243–244
Genetic manipulation of *Mortierella alpina* strains, 28–29
Genetics of *Ulkenia,* 102–104
Geotrichum candidum, biomass production with, 3
GLA-Forte, 6
GLA-single cell oil process, 5–7, 204
Glycerophospholipids (GPL), presence of, in brain, 182

H

Haematococcus pluvialis, as source of astaxanthin, 127–129, 232–233
Heterokonta, 43
Heterotrophic cultivation systems, 149
Heterotrophic production of docosahexaenoic acid, 107–121
Hexane, solvent extraction using, 203
High-oleic sunflower oil (HOSO), 95
Human health, importance of long-chain n-3 fatty acids in, 36–37
Human milk, long-chain polyunsaturated fatty acid (LCPUFA) in, 73
Hydraulic pressing, 211
Hygromycin resistance, 28–29

I

Infant formulas
adding long-chain polyunsaturated fatty acid to, 186
arachidonic acid in, 9–10, 73–74
single cell oils in, 188–189, 244
International Society for the Study of Fatty Acids and Lipids (ISSFAL), 88
Ischemia, 185

K

Kelco Biopolymers, 43
Kinetic aspects of supercritical fluid extraction, 224–228

L

Labyrinthula, 12
Labyrinthulomycota, 100
Leukotrienes, 138
Lindner, Paul, 1
Linoleic acid (LA), 24
conversion of oleic acid to, 140
γ-Linolenic acid oil (GLA)
borage oil as source of, 6
as component of evening primrose oil, 5
extraction from *Mortierella isabellina,* 240
extraction from *Mucor circinelloides,* 204–205, 240
fatty acid profiles of fungi and plants used commercially for, 6–7
production of, 5–7
Lipids
accumulation in an oleaginous microorganism, 2–3
composition of *Parietochloris incisa,* 60–61
production from C_2 carbon sources, 120–121
supercritical fluid extraction of, from algae, 220–235
Lipoxygenase, 185
Long-chain polyunsaturated fatty acids (LCPUFA)
biosynthesis of, 76–77
in *Mortierella alpina,* 78–79
importance of, in human health, 36–37
sources of, 37–38
trials involving, in preterm infants, 189–190
Lutein, 124
potential algal sources of, 129
Lycopene, 124

M

Marine fish oil as source of eicosapentaenoic acid, 138
Martek Ltd, 9, 10, 11
Mead acid, production of, 13
Mercury compounds in fishmeal, 12
Microalgae
accumulation of PUFA in, 54–55
cultivation strategies for eicosapentaenoic acid production by, 149–150
cultivation systems for, 147–149
eicosapentaenoic acid (EPA) production by, 144–153
extraction of oils from, 213–216
occurrence of PUFA-rich TAG in, 53–54

as source of eicosapentaenoic acid, 142–143
as source of n-3 PUFA, 99–100
Microbes, genetic engineering of, 242–243
Microbial fat, unease of using in chocolate products, 8
Microbial oils
development of efficient large-scale production of, 4
early interests in producing, 4–5
work on production of, 2
Microbial protein, 4
Microorganisms
carotenoid production using, 124–131
as source of eicosapentaenoic acid, 142–143, 150–153
Monodus subterraneus as source of EPA, 213
Monogalactosyldiacylglycerol (MGDG), 55, 57, 65, 66, 67
distribution and content of, in *Parietochloris incisa,* 67
proportion of eicosapentaenoic acid in, 59
Monsanto, 16
Mortierella, extraction of arachidonic acid from, 73–81, 234–235, 241
Mortierella alliacea, 12
Mortierella alpina, 10, 13, 74
analysis of enzyme genes involved in PUFA biosynthesis, 26–28
derivation of mutants from, 23–25
extraction of arachidonic acid from, 206–208
fermentation of, 80–81
genetic manipulation of strains of, 28–29
long-chain polyunsaturated fatty acid (LCPUFA) biosynthesis in, 78–79
properties of, 74–78
single cell oils produced by, 165
as source of eicosapentaenoic acid, 142
Mortierella isabellina, 6
extraction of γ-linolenic acid oil from, 240
Mortierella ramanniana, 6
Mucor circinelloides, 6
extraction of single cell oil γ-linolenic acid from, 204–205, 240
production of single cell oils using, 5–6
Mutants
derivation of, from *Mortierella alpina* 1S-4, 23–24
with enhanced desaturase activities, 25
selection of chill-sensitive, of *Porphyridium cruentum,* 57–59
Mutation in improving microalgal strains, 153
Mutual recognition, principle of, 175

N

NADH-cytochrome b_5 reductase, 28
Nagase-Suntory Co. Ltd, 13
Nannochloropsis sp.
production of eicosapentaenoic acid from, 148–149
as source of EPA, 213
Neochloris wimmeri, 129
Nicotinamide adenine dinucleotide phosphate (NADPH), generation of, 14–17
Nitrogen starvation, 2–3, 55, 214
accumulation of fatty acids following, 2–3, 62
enhancement in content of arachidonic acid under, 61
recovery from, 58–60
Nitzschia laevus, eicosapentaenoic acid in, 143, 146
Nonclinical toxicology of single cell oils, 166–168
Novel foods, 172, 173–174
Novel Foods Regulation, 161, 174, 175, 176
Nutritional aspects of single cell oils, 182–192
Nutritional factors, influence on eicosapentaenoic acid production, 145–146

O

Octadecadienoic acid, cultivation of, 23
Odontella aurita
eicosapentaenoic acid in, 143
marketing of, as novel food, 175

Oidium lactis (now *Geotrichum candidum*), biomass production with, 3
Oil accumulation
in microalgae, 54
in oleaginous microorganisms, 2
Oil extraction processes, 204–213
Oil of Javanicus, 6
Oleaginous microorganisms
accumulation of lipid in, 2–3
oil accumulation in, 2
Oleic acid (OA)
conversion of, to linoleic acid (LA), 140
cultivation of, 23
de novo synthesis of, 140
OmegaTech Ltd, 10, 11
Open pond systems, 126, 143, 147–148

P

Palmitic acid, conversion to stearic acid, 25
Parietochloris incisa, 12
biosynthesis of arachidonic acid in, 64–66
extraction of arachidonic acid from, 235
induced accumulation of arachidonic acid in, 61–63
isolation and characterization of, 60–61
lipid composition of, 60–61
role of arachidonic acid in, 66–68
Penicillium chrysogenum, cultivation of, 4
Perfusion culture, 150
pH, influence on eicosapentaenoic acid production, 147
Phaeodactylum tricornutum
conversion into heterotroph, 243
production of eicosapentaenoic acid from, 148–149
in production of n-3 PUFA by, 147
as source of eicosapentaenoic acid, 143, 146, 213
Phaffia rhodozyma, extraction of astaxanthin from, 233–234
Phaffia rhodozyma/Xenophyllomyces dendrorhous as source of astaxanthin, 129
Phauxostat, 110
pH auxostat, 110
Phosphate, role in energy transfer of cells, 146
Phosphatidylethanolamine (PE), 182
in *Parietochloris incisa,* 60–61
Phosphatidylserine, 182
Phospho-proteolipids, 43
Photoautotrophic cultivation systems, 147–149
Photobioreactors in production of eicosapentaenoic acid, 148
Plants, genetic engineering of, 243–244
Polar modifier, 224
Polychlorobiphenyls (PCB), in fishmeal, 12
Polyketide synthase systems, 44–45
Polyunsaturated fatty acid (PUFA), 234
accumulation of, in microalgae, 54–55
alternative pathway for synthesis of, 44
analysis of enzyme genes involved in biosynthesis of, in *Mortierella alpina,* 26–28
biosynthesis of, in *Porphyridium cruentum,* 55–56
cellular content of, 144
dietary significance of very long-chain, 1
microalgae as source of, 99–100
PUFA-rich TAG as reservoir of, for modification of choloroplastic lipids, 59–60
in tissues and functional studies, 186–188
Polyunsaturated fatty acid (PUFA)-rich TAG
occurrence of, in microalgae, 53–54
use of, as reservoir of PUFA for modification of choloroplastic lipids, 59–60
Polyunsaturated fatty acid (PUFA)-single cell oils
clinical application of, 13–14
sources of, 10–14
Porphyridium cruentum
biosynthesis of PUFA in, 55–56
molecular species composition of galactolipids of, 56–57
production of, 54
selection of chill-sensitive mutants of, 57–59
as source of EPA, 213

Porphyridium purpureum
eicosapentaenoic acid in, 143
optimal temperature for growth of, 147
Postmarket surveillance, safety in, 168–169
Poultry feed, incorporation of *Schizochytrium* biomass into, 12
Preterm infants, trials involving long-chain polyunsaturated fatty acid in, 189–190
Prokaryotic lipids, 55–56
Prostaglandins, 138
Protosiphon botryoides, 129
Prymnesium toxin, 43

R

Rapeseed (Canola) oil, 2
Reactive extraction, 227
Redbook, 162
Regulatory requirements for food ingredients, 169–171
Respiratory Quotient (RQ), 80–81
Reversed-phase chromatography, 216
Rhodotorula glutinis, 129
Risk analysis, 161
Risk assessment, 161
Risk communication, 161
Risk management, 161

S

Safety
of biomass and extracted DHA-rich oil, 46–47
defined, 161
of food additives, 163
of food ingredients, 162–163
of single cell oils, 161–162, 166, 188
of source organisms, 164–166
Safety assessment approach, 161, 163–164
Safety regulations
in Europe, 172–175
in United States, 169–172
Salicylhydroxamic acid (SHAM), 66
Scale-up of the fermentation technology, 43–46
Schizochytrium, 243
development of a docosahexaenoic acid production technology using, 36–48
docosahexaenoic acid (DHA) oil from, 241
research involving, 45
Schizochytrium oil, 10–11
marketing of, 11
Schizochytrium sp., 100
docosahexaenoic acid oil derived from, 165
extraction of DHA-rich oils from, 208–210
toxicological assessment of, 168
Schizophrenia, 185
Shewanella proteins, 44–45
Single cell oils (SCO), 1–17
clinical studies of, 168
in adults, 190–192
in infants, 188–189
commercial production of, 239–241
competition from genetically modified plant oils and, 14–17
defined, 1
early interests in, 1–8
Europe Union approval for, 175
extraction of, from microalgae, 213–216
extraction process for, 202–213
future uses of, 244–246
GRAS status of, 171–172
for human consumption, 164–166
Mortierella as source of, 21–22
new sources of, 241–242
nonclinical toxicology of, 166–168
nutritional aspects of, 182–192
production of, by dinoflagellates, 86–97
safety of, 161–162, 166, 188
Single cell protein (SCP) production, 4
Solvent extraction, 211
using hexane, 203
Source organisms, safety of, 164–166
Soybean oil, 2
Sphingolipids, presence of, in brain, 182
Sphingomyelin, presence of, in brain, 182
Starflower oil, 204
Static extraction mode, 225

Stearic acid
almitic acid conversion to, 25
in production of cocoa butter equivalent fat, 7
Stearidonic acid, production of, 13
Streptomyces mediolani, 131
Substantial equivalence concept, 174, 176
Supercritical fluid
carbon dioxide as preferred, 222–224
extraction of lipids and other materials from algae, 220–235

T

Temperature
influence on eicosapentaenoic acid production, 146–147
modification of molecular species composition of galactolipids of *Porphyridium cruentum,* 56–57
Thermodynamic aspects of supercritical fluid extraction, 224–228
Thraustochytriales, 100
Thraustochytrids, 40–41
Thromboxanes, 138
Tissues, polyunsaturated fatty acid levels in, 186–188
Torularhodin, 129
Torulene, 129
Total fatty acids (TFA), arachidonic acid in, 53, 54
Triacylglycerols (TAG), 53, 74
plant accumulation of, 68
selection of chill-sensitive mutants of *Porphyridium cruentum* and, 57–59
Triarachidonylglycerol, 62
Trichosporon pullulans, 1
Tuna oil, 9

U

Ulkenia, 12, 165–166
genetics of, 102–104
mass cultivation of, 100–101
production of docosahexaenoic acid by, 99–105
as source of docosahexaenoic acid, 100, 101–102, 105
United States, safety regulations in, 169–172

V

Very long-chain polyunsaturated fatty acids (VLCPUFA) 10, 11
abundance of, in microalgae, 53
successful genetic engineering of, 14
utilization of, 68

W

Wet extraction processes, 220
World Health Organization (WHO), 88
Wuhan Alking Bioengineering Co. Ltd, 12

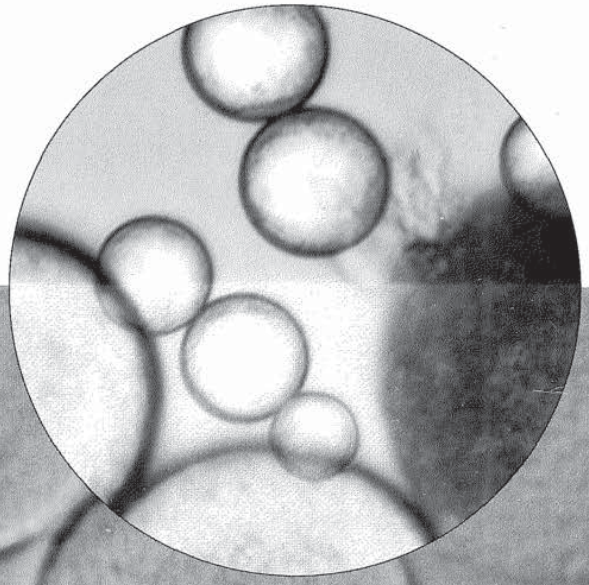
Y

Yarrowia lipolytica, 4
Yeasts, production of cocoa butter equivalent fat from, 7–8

Z

Zeaxanthin, 124, 131
Zygomycetes, 5, 10

Thraustochytrids, a source of single cell oils, show great morphological diversity from single cells to clusters of cells, complex reticulate forms, and motile stages. The organism shown was isolated recently from temperate Australian waters (2004). Photograph supplied by Dion Frampton of CSIRO Marine Research, Hobart, Tasmania, Australia. Used with permission of CSIRO.



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