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# REVIEW

# Biosynthesis and Regulation of Microbial Polyunsaturated Fatty Acid Production

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**Growing interest in polyunsaturated fatty acid (PUFA) applications in various fields coupled with their significance in health and dietary requirements has focused attention on the provision of suitable sources of these compounds. Isolation of highly efficient oleaginous microorganisms has led to the development of fermentation technologies as an alternative to agricultural and animal processes. Particularly active in PUFA synthesis are the Zygomycetes fungi and certain microalgae. Emphasis is placed on increasing the product value by employing new biotechnological strategies (e.g. mutation techniques, molecular engineering and biotransformations) which allow the regulation of microbial PUFA formation with satisfactory yield in order to be competitive with other sources. Comparative successes in fungal PUFA production demonstrate microbial potential to synthesize high-value oils and provide the main stimulus for their applications.**

**[Keywords:** microorganisms, polyunsaturated fatty acids, regulation, overproduction]

Microorganisms have often been considered for the production of oils and fats as an alternative to agricultural and animal sources. However, owing to their resemblance to plant oils and animal fats, microbial oils will inevitably have to compete with traditional lipid products if they are to be produced commercially. It is clear that with the lowering cost of plant oils and animal fats, there is no prospect that microbial oils similar to these could ever be produced economically. Therefore, if microorganisms are to be thought of as sources of single cell oils, then their oils must be highly specific and currently expensive to obtain from agricultural and animal sources, or not commonly synthesized by these sources. Although amazing diversity of fatty acid structures occurs in the microbial kingdom (1), many of the minor fatty acids have potential uses but are not available in sufficiently large quantities. Increasing demand for commercially valuable lipids has resulted in production of PUFAs for health and dietary applications. This review deals with the biosynthesis of microbial PUFAs with emphasis on their regulation and overproduction.

## IMPORTANCE AND SOURCES OF PUFAs

PUFAs, with their unique structural and functional characteristics, are distinguished by two main functions (2). The first function relates to their roles in regulating the architecture, dynamics, phase transition and permeability of membranes, and modulating the behaviour of membrane-bound proteins such as receptors, ATPases, transport proteins and ion channels. In addition, PUFAs control the expression of certain genes (3) and thus affect some processes including fatty acid biosynthesis and cholesterol transport in the body. Much more interest is focused on the second role of PUFAs as precursors of a wide variety of metabolites (such as prostaglandins, leukotrienes and hydroxy-fatty acids) regulating critical biological functions. The various roles played by PUFAs make it apparent that they are required in every organ in the body in order for the organs to function normally. Therefore, it is not surprising that PUFA deficiencies lead to abnormalities in the skin, nervous system, immune and inflammatory systems, cardiovascular system, endocrine system, kidneys, respiratory and reproductive systems. Because mammals lack the ability to synthesize PUFAs, these must be supplied in the diet. Moreover, PUFAs also have a number of uses in various fields and the biomedical and nutraceutical applications of commercial PUFA-rich preparations have been recently reviewed by Gill and Valivety (4).

The main oil sources relatively rich in $C_{18}$ PUFAs are the seeds of some plants with dominant synthesis of linoleic acid (18 : 2 *cis* 9,12) and ALA. Occurrence of significant levels of essential GLA in plants is rare and the most commercially important sources of this fatty acid are seeds of evening primrose *(Oenothera biennis)* with 8-10% (w/w) GLA, borage seeds *(Borago officinalis)* containing 24-25% (w/w) GLA and black currant seeds *(Ribes nigrum),* which consist of 16-17% (w/w) GLA (5) (Table 1). In contrast, PUFAs above $C_{18}$ cannot be synthesized by higher plants in any significant amounts owing to a lack of the requisite enzymes. On the other hand, the beneficial affect of fish oil has been ascribed to its $C_{20}$ and $C_{22}$ w-3 fatty acid content, notably, EPA, DPA-$\omega$3 and DHA (6). However, fish oils possess objec-

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Abbreviations: AA: arachidonic acid (20:4 *cis* 5,8,11,14); ALA: a-linolenic acids (18 : 3 *cis* 9,12,15); DGLA: dihomo-$\gamma$-linolenic acid (20: 3 *cis* 8,11,14); DHA: docosahexaenoic acid (22 : 6 *cis* 4,7,10, 13,16,19); DPA-w3: docosapentaenoic acid (22 : 5 *cis* 7,10,13,16, 19); DPA-$\omega$6: docosapentaenoic acid (22 : 5 *cis* 4,7,10,13,16); EPA: eicosapentaenoic acid (20 : 5 *cis* 5,8,11,14,17); ETA: eicosatetraenoic acid (20 : 4 *cis* 8,11,14,17); GLA: $\gamma$-linolenic acid (18 : 3 *cis* 6,9,12); MA: mead acid (20 : 3 *cis* 9,12,15); PUFA: polyunsaturated fatty acid; SSF: solid state fermentation; TAG: triacylglycerol.

TABLE 1. Sources of polyunsaturated fatty acids

| PUFA | Conventional sources | Microbial sources | Ref. |
|---|---|---|---|
| GLA | **Plants** (Evening primrose, Borage, Blackcurrant) | **Fungi** ***(Mucor circinelloides, Mucor mucedo, Mortierella isabellina, Mortierella ramanniana, Cunninghamella echinulata, Cunninghamella elegans,*** *Cunninghamella japonica, Rhizopus arrhizus, Thamnidium elegans),* **algae** ***(Spirulina platensis, Chlorela vulgaris)*** | 15, ***16,*** 17, 18, 58, 76 |
| DGLA | **Human milk, animal tissues, fish (*Scomber scrombrus*), mosses** ***(Pogonatum urnigerum)*** | **Fungi** *(Mortierella* spp., especially *Mortierella alpina, Conidiobolus nanodes,* ***Saprolegnia ferax)*** | ***2,*** 1, ***8,*** 13, ***37, 57*** |
| AA | **Animal** *tissues, fish (Brevoortia, Clupea),* **mosses** *(Ctenidium* ***molluscum)*** | **Fungi** *(Mortierella* spp., especially *Mortierella alpina, Conidiobolus nanodes,* ***Entomophthora exitalis, Blastocladiella emersonit),*** **algae** ***(Porphyridium cruentum,*** *Sargassum* ***salicifolium, Euglena gracilis)*** | ***2, 7, 13,*** 14, ***37, 57, 58,*** 76 |
| MA | **Animal tissues** | **Fungi** *(Mortierella alpina)* | 109, 37, 110 |
| EPA | **Fish** (herring, menhaden), **shellfish** (blue crab, oyster, lobster, mussel) | **Fungi** ***(Mortierella alpina, Mortierella elongata, Pythium irregulare, Pythium ultimum),*** **algae** ***(Chlorella minutissima, Chlorella*** *minitissima,* ***Monodus subterraneus,*** *Polysiphonia* ***latifolium, Porhyridium cruentum, Phaeodactylum tricornutum, Nannochloropsis oculata, Amphidinium carteri,*** *Thalassiosira pseudonana),* **bacteria** ***(Shewanella putrefaciens)*** | 6, 7, 11, 37, 90, 91, 97 99, 100 |
| ETA | **Animal tissues** | **Fungi** *(Mortierella alpina)* | 107, 108 |
| DPA | **Fish** | **Fungi** *(Schyzochytrium* sp.) | 6, 106 |
| DHA | **Fish** (tuna, herring, cod, sardine, salmon, menhaden), ***shellfish*** (blue crab, mussel, lobster, oyster) | **Fungi** ***(Thraustochytrium aureum, Thraustochytrium roseum, Schyzochytrium*** **SR21,** *Schyzochytrium aggregatum),* **algae** (microalgae MK8805, ***Crypthecodinium cohnii, Gyrodinium nelsoni, Amphidinium carteri, Gonyaulax*** *polyedra),* **bacteria** *(Vibrio* spp., *Rhodopseudomonas* spp.) | ***6,*** 11, 102, 103 |

tionable tastes, and odours and their satisfactory utilization requires the removal of cholesterol and small amounts of potentially toxic impurities (e.g. pollutants). There are also additional drawbacks to the use of fish oils as a PUFA source, such as variations in oil quality and the presence of fatty acids with antagonistic properties (e.g. arachidonic acid). Total content of $\omega$-3 fatty acids depends considerably on the season and geographic location of harvest sites as well as on the species of fish and the type and availability of its primary food source, namely marine microorganisms. Furthermore, it has been predicted that if w-3 PUFAs come to be widely used as prophylactic drugs, the total present annual production of marine fish oil would be insufficient to meet the worldwide demand.

Oleaginous microorganisms, as alternatives to agricultural and animal oil products, have also been intensively studied. Certain fungi, marine bacteria, heterotrophic and phototrophic microalgae and mosses contain various PUFAs, and may thus represent suitable sources of them (7-11). The diversity of microbial species can facilitate the selection of strains producing oil with predominant fatty acid. Although the contribution of microbial lipids to the oil industry is nearly negligible, there are several reasons to be enthusiastic about them: (i) active lipid-synthesizing apparatus makes oleaginous microorganisms perspective oil sources; (ii) microbial PUFAs are the higher value oil types rather than resembling the bulk of low-priced commodities such as soybean oil, palm oil and sunflower oil; (iii) their extremely high growth rates on wide varieties of substrates allow to utilize cheap or zero-cost materials; (iv) oil production can be carried out throughout the year, there is no seasonal or climatic dependence; (v) microbial sources can supply more concentrated pharmaceutical-grade PUFAs than other sources with controlled quality; (vi) microbial competence to carry out numerous transformation reactions (e.g. oxidation, desaturation and hydrogenation) enables the upgrading of PUFA structures and allows simultaneous formation of lipid and other products; (vii) existence of numerous mutants defective in specific enzymes (e.g. desaturases) improves production of tailor-made oils; (viii) oleaginous microbes can be considered as the appropriate hosts into which foreign (plant or animal) genes could be cloned for the production of desired fatty acids hitherto not found in microorganisms but which occur in other oil and fat sources; (ix) microorganisms provide useful models for studying lipid biochemistry, metabolic control and function because the fewer organelle compartments in their cells allow to answer the key metabolic and biochemical questions more simply and faster than in complex multicellular systems of other organisms; (x) due to simplicity of microbial metabolic regulation, they can be readily grown under controlled conditions with nutritional regimes that may stimulate or repress the key steps of fatty acid formation and allow the manipulation of the lipid yield and profile; (xi) microorganisms are rich in protein, trace elements, vitamins, antioxidants, etc. so they could be employed as macro- and micronutrients.

## PUFAs AS TAXONOMIC CRITERION

A variety of PUFAs have been detected in microorganisms including bacteria, fungi, algae, mosses and protozoa (1) and their cellular fatty acid composition has been used to characterize, differentiate and identify various genera, species and strains. The microbial fatty acid composition offers valuable taxonomic and physiologic information as to its relationship with other microorganisms that may not be available from morphologic characteristics. Thus, physiological data provided by fatty acid profiles, when used in conjugation with morphological characteristics, have the potential to increase the accuracy and resolution of sorting large numbers of microbial isolates. This information is especially useful at the subspecies level, where morphological disparities are limited. For example, fatty acid analysis of several vesicular-arbuscular mycorrhizal fungi tentatively classified as Zygomycetes, has revealed that these fungi have a fatty acid profile more similar to that of Protoctisan fungi (12). Also, the PUFA composition appeared to be a help-

ful chemotaxonomic marker for the genus ***Mortierella*** at the subgeneric level, where two subgenera were clearly distinguished from each other (13, 14). Whilst ***Mortierella*** subgenus ***Micromucor*** produced only PUFAs with maximal length of $C_{18}$ carbon chain, presence of $C_{20}$ PUFAs was characteristic for ***Mortierella*** subgenus ***Mortierella.*** On the other hand, although the fatty acid profile may be a reliable technique and criterion to characterize species and strains, the current deviations in one genus were found. For example, previous studies have revealed intra-genus variability of the fatty acid composition in *Mucorales* (15-19). This diversity of microbial lipid composition is primarily determined by activities of the key enzymes of lipid metabolism, which reflect the physiological status of strains under cultivation conditions. Certik and Sajbidor (18) concluded that mainly PUFAs with high intra-genus variability are involved in adaptation changes to secure the optimal fluid properties of cell membranes.

## BIOSYNTHESIS OF MICROBIAL PUFAs

The prospect for efficient microbial PUFAs production requires knowledge of the mechanisms by which their biosynthesis is accomplished within the cell. These are, however, still poorly understood, although the main aspects of PUFA accumulation are known. There are several distinct pathways that could be involved in this biosynthetic process (20): (i) ***de novo*** synthesis of fatty acids from glucose; (ii) the incorporation of exogenous fatty acids directly into lipid structures; and (iii) following desaturation and elongation of lipid sources. In addition, fatty acid biohydrogenation (saturation) and partial or total degradation (P-oxidation) also contribute to this process.

Unsaturated fatty acids are synthesized by either an aerobic or an anaerobic mechanism. PUFA synthesis is associated with membrane-bound enzymes and the desaturation system of the aerobic pathway is composed of three proteins: NAD(P)H-cytochrome $b_5$ reductase, cytochrome $b_5$ and the terminal cyanide-sensitive desaturase (Fig. 1). There are three types of desaturases: (i) acyl-CoA desaturases, (ii) acyl-ACP desaturases, and (iii) acyl-lipid desaturases. Generally, stearoyl-CoA or stearoyl-ACP is the substrate for the first double bond introduction to form oleoyl-CoA or oleoyl-ACP, respectively. Subsequent desaturation takes place in the endoplasmic reticulum where fatty acids bound to phospholipids (especially phosphatidylcholine) are desaturated rather than thiol CoA esters (21, 22). However, besides this dominant desaturation pathway, direct conversion of fatty acyl-CoA to the corresponding PUFA is also found in the endoplasmic reticulum (21).

The first double bond in the aerobic pathway dominant in eukaryotic cells and some bacteria is invariably introduced into the $\Delta^9$ position of saturated fatty acids, thus, palmitoleic (16: 1 ***cis 9)*** and oleic (18 : 1 ***cis*** 9) are the most common monoenes in microorganisms. Oleic acid is then, in general, desaturated by $\Delta^{12}$ desaturase to yield linoleic acid, which may be further converted by $\Delta^{15}$ desaturase to a-linolenic acid. Thus, these three fatty acids are the basic precursors of the w-9, o-6 and w-3 fatty acid cascades (Fig. 2). The next steps for PUFA production are desaturation of appropriate fatty acid precursors by $\Delta^6$ desaturase followed by successive chain elongations and subsequent desaturation(s) to yield the respective $C_{20}$ and $C_{22}$ PUFAs. The w-9 family of PUFA is synthesized from oleic acid and sequential participation of $\Delta^6$ desaturase, elongase and $\Delta^5$ desaturase to finally produce MA. The w-6 type of fatty acids is usually formed from linoleic acid via desaturation ($\Delta^6$, $\Delta^5$, $\Delta^4$) and elongation steps from linoleic acid through GLA, AA and adrenic acid (22 : 4 ***cis 7,*** 10,13,16) to DPA-$\omega$6. Finally, two routes operate for o-3 PUFAs synthesis in microorganisms (23). In the first route, which is temperature independent, ALA is metabolized via the o-3 pathway to EPA, DPA-$\omega$3 and DHA. The second temperature dependent route involves the conversion of $\omega$-6 fatty acids into corresponding w-3

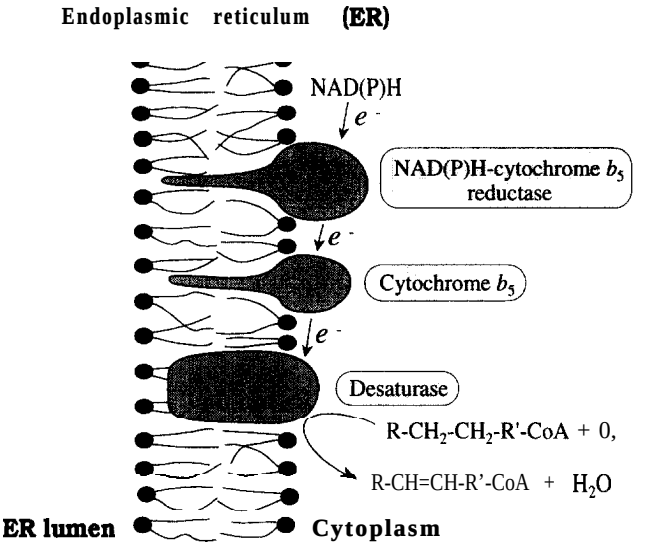


FIG. 1. Aerobic desaturation complex of unsaturated fatty acids formation in microorganisms.

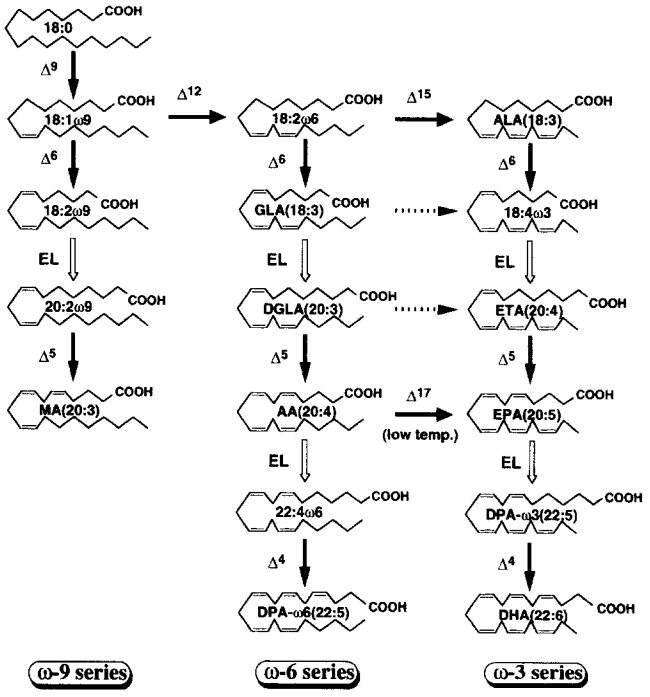


FIG. 2. Biosynthetic pathways of polyunsaturated fatty acids in microorganisms. EL-elongase; $\Delta^4$, $\Delta^5$, $\Delta^6$, $\Delta^9$, $\Delta^{12}$, $\Delta^{15}$ and $\Delta^{17}$-desaturases.

PUFAs catalyzed by two possible enzymes, $\Delta^{15}$ and $\Delta^{17}$ desaturases ($\omega^3$ desaturases).

In addition, differences in the PUFA formation between ***in vivo*** and ***in vitro*** systems of *Mortierella ramanniana* were observed (24). While intact cells of the strain sequentially desaturated oleic and linoleic acids into GLA, free membrane bound enzymes formed hydroxylated fatty acids. Although membrane desaturases have many superficial similarities to the hydroxylases (25) and certain desaturations might take place in two steps (hydroxylation by molecular oxygen, followed by dehydration of the hydroxy derivative), further studies are necessary to shed more light on the relationship between these two pathways.

Once desaturation has occurred, the acyltransferation reactions then facilitate distribution of newly synthesized PUFA to other cellular lipids. Many microorganisms accumulate large amounts of PUFAs as storage lipids in the form of triacylglycerols which are synthesized from *sn*-glycerol-3-phosphate and acyl-CoA through a sequential process (known as the Kennedy pathway) involving glycerol 3-phosphate acyltransferase, lysophosphatidate acyltransferase, phosphatidate phosphatase and diacylglycerol acyltransferase (26, 27). However, the assembly of three fatty acids onto the glycerol backbone is not always as straightforward as outlined in the Kennedy pathway and most fatty acids are not immediately available for TAG biosynthesis. As mentioned above, the acyl chains are bound to phospholipids where they become desaturated or otherwise modified. Fatty acids from phospholipids are then available for TAG biosynthesis by one of two mechanisms responsible for regulation of TAG unsaturation (28). In the first, acyl exchange occurs between acyl-CoA pool and acyl-phospholipid (mainly phosphatidylcholine) by the combined reverse and forward reactions of acyl-CoA:phospholipid acyltransferase. The resulting newly formed PUFA-CoAs enrich the acyl-CoA pool where they serve as acyl donors in TAG synthesis via the Kennedy pathway. The second mechanism involves donation of the entire diacylglycerol portion from phosphatidylcholine catalyzed by reversible CDP-choline phosphotransferase and these diacylglycerol moieties with newly modified PUFAs may be directly available for TAG synthesis. Thus, acyltransferases and desaturases may be tightly bound and their cooperation including their specificities and selectivities to different acyl-CoA species is responsible for PUFA distribution among individual lipid structures.

## PROCESSES FOR MICROBIAL PUFA PRODUCTION

The extensive research and development of PUFA production carried out over the past several years is still continuing and is basically aimed at improving the economic competitiveness of microbial lipids compared to plant- and animal-derived oils. Emphasis is put on increasing the product value, using inexpensive substrates, screening for more efficient strains and reducing the processing steps necessary for oil recovery from the cells. Screening of potential microorganisms for PUFA production is the first and an essential step limiting the number of strains for further study and practical use. These strains can be directly used in laboratory-scale optimization process. Since the entire pathway of microbial lipid biosynthesis is under strong regulatory control, mathematical models determining the relationship between availability of various nutrients and oil concentrations and their composition in the cells have been applied to the process to achieve the maximal PUFA yield (29). Two basic processes have been developed for microbial production of PUFAs: submerged and solid state fermentations. Simultaneously with fermentation trials, toxicological treatments of the strains and their metabolites must be performed to determine the safety of the product (30, 31).

**Submerged fermentations** Submerged cultivations for industrial use require a process that consists of several operation units from strain cultivation to oil refining. There are, however, three key operations which require special attention to the process development owing to the nature of oleaginous organisms: (i) fermentation; (ii) cell separation; and (iii) oil extraction and refining. Because the economic considerations of PUFA production are severely affected by the cost of raw material feedstock, selection of growth media (usually nitrogen-limited) is an important step. It should be noted that media adequate for the screening process are different from large-scale production media. Under optimal conditions in a fermentor, microorganisms require 5 kg of fermentable substrate to produce 1 kg of TAG-type oil. The most cost efficient substrates are the waste materials from the food industry, preferable when the oil is intended for human consumption. After appropriate pretreatment, the substrate is either batch or continuously fermented under favourable conditions with sufficient amount of inoculum to yield biomass rich in PUFA-oil.

**Solid state fermentations** The principal difficulty that has been experienced with submerged PUFA production has been in its marketing rather than in developing the large-scale fermentation and oil extraction processes. The association of the fungal oil with SSF provides another opportunity to fill marketing claims. Because these fungi simultaneously decrease anti-nutrient compounds in the substrates (e.g. phytic acid) and partially hydrolyze substrate biopolymers, prefermented mass with a high content of PUFAs may be used as inexpensive food and feed supplement (Slugen, D. ***et al.,*** Czech Patent Appl. 279043, 1994). However, many scale-up problems of SSF processes employed in PUFA production have to be solved before a laboratory-scale process can be transferred to the commercial level. There are fewer studies on the factors influencing the microbial growth and lipid formation in large-scale SSF than those with submerged fermentation. The two most important factors are mass transfer (including both transfer of oxygen to the growing microorganisms and transfer of nutrients and enzymes within the substrate solid mass) and removal of reaction temperature generated during fermentation. In general, development of a desirable SSF process for fungal PUFAs involves many steps that are as follows: (i) isolation, screening and selection of an appropriate microorganism, (ii) optimization of physicochemical and nutritional parameters as well as standardization of process unit operation on a laboratory scale, (iii) scale-up studies and, if necessary, the design and establishment of the pilot plant, (iv) generation of engineering data, design and layout of the commercial plant, (v) construction of the plant and solving any difficulties in plant operation during the commissioning step, and finally (vi) regular operation of the plant for the production of microbial metabolite(s). Scale-up is

therefore the crucial link in the transfer of laboratory-scale processes to commercial production scale and brings several problems such as variations in the biomass formed, large-scale inoculum development, medium sterilization, aeration, agitation, maintenance of constant heat and water balance, pH control, contamination control, heterogeneity, downstream processing, water and solid waste handling (32). Nevertheless, PUFA production by SSF could be a useful method for a newly developing market, as the risk to producers and the investment cost may be expected to be less dramatic.

## REGULATION AND CONTROL OF MICROBIAL PUFA FORMATION

Comparative success in microbial oil production has led to a flourishing interest in the development of fermentation processes and has enabled several processes to attain commercial production levels (4). However, two main problems still exist - the economic and marketing difficulties. On the other hand, the cost of microbial oil may be secondary compared to the health (functional, dietetic and therapeutical) benefits derived. Nevertheless, microbial oils must yield to the best commercial varieties and have a broad production base to be competitive with other commodities. Although the manipulation of microbial oil composition is a rapidly growing field of biotechnology, the supply of microbial lipids is still insufficient to meet industrial demand. Therefore, alternative strategies (for example mutation methods, molecular engineering techniques, the use of inhibitors, structuring microbial fatty acid composition by enzymatic treatment of preexisting oils) should be combined with classical fermentations.

**Mutation techniques** Although several wild-type oleaginous microorganisms are able to synthesize PUFA-rich oils, these strains have a limited ability to produce new PUFAs or increase existing PUFA formation. Mutation techniques resulting in the suppression or activation of specific desaturases and elongases are beneficial not only for the production of tailor-made fatty acids, but they can also be useful for studying fatty acid biosynthesis in a microbial body (23). Several mutants with defective desaturases ($\Delta^5$, $\Delta^6$, $\Delta^9$, $\Delta^{12}$ and $\omega^3$ [$\Delta^{15}$, $\Delta^{17}$]), enhanced desaturase activities ($\Delta^5$, $\Delta^6$) or their combinations (33-37) as well as elongation defective mutants (38) have been characterized and employed for PUFA formation. These mutants not only exhibit significantly improved production of naturally occurring PUFAs, but also form other PUFAs commonly not found in the wild-type microorganisms. Moreover, the ability of these mutants to utilize exogenous fatty acids allows the production of various PUFAs in high yield. This phenomenon is very promising from the biotechnological point of view because there are various sources of easily available natural oils containing individual fatty acid precursors. Thus, the mutants are excellent tools for regulating exogenous fatty acid flow to targeted PUFAs. Moreover, due to simplicity of their metabolic system, they are excellent models for elucidating the reaction mechanisms involved in fatty acid biosynthesis. Among them, the mutants of ***Mortierella alpina,*** with their unique fatty acid biotransforming enzyme systems, are probably the best studied microbial strains (23, 37).

**Molecular engineering microbial lipids** Progress in the genetic engineering of microorganisms has led to speculation that certain oil components could become marketable commodities if the genomes of traditional oleaginous microbes were appropriately modified. The ideal oil for commercial-scale application would consist of a particular type of PUFA that could be supplied constantly at a competitively low price as compared with raw or currently used materials. However, microbial fatty acids biosynthesis requires the interaction of multiple gene products. To what degree must the lipid synthetic apparatus be modified to yield industrially applicable changes, remains to be determined.

A major challenge in modifying the lipid composition is to change the degree of fatty acid unsaturation and to reduce or increase the chain length of fatty acids. However, most of the enzymes that modify the structure of microbial fatty acids are membrane bound, which has significantly hindered efforts to purify and study their action. From this point of view, molecular engineering provides very powerful methodology and tools not only for the investigation of complex metabolic pathways involved in PUFA formation, but also for the production of novel microbial varieties synthesizing economically valuable fatty acids. Because biochemical mechanism(s) and the structure/function relationship of the fungal desaturases, such as positional specificity or the relationship between desaturases and other fatty acid modifiers, are still not well understood, a rational strategy for isolation and expression of the genes responsible for the desired metabolic steps have to be designed. There are three interdependent genetic technologies to engineer fatty acid composition: (i) cloning of genes encoding proteins involved in PUFA biosynthesis; (ii) transgenic expression of these genes; and (iii) modification of cloned genes in order to engineer the expressed protein (39).

Progress in this area has been mainly advanced in plants and cyanobacteria (39, 40), where many of the soluble and membrane-bound enzymes involved in fatty acid unsaturation have been characterized and cloned. Most of the already cloned genes have significant homology to their microbial or animal counterparts. Thus, genes isolated from one organism could be transferred into microorganisms or to used as heterologous probes for the isolation of appropriate microbial genes and finally to alter fungal oils. By attaching the plant (41–43), human (44) or animal (45) clone to a suitable microbial promoter and microbial subcellular targeting sequences, it may be possible to modify the chain length or amount and position of double bonds in fatty acids produced in microbial bodies as well as conduct structure/function studies through the use of site-directed mutagenesis. Conversely, the introduction of microbial lipid biosynthetic genes into specific subcellular locations of plants (46, 47) and human cells (48) also provides a useful tool for uncovering reactions and components of the key regulatory systems in these organisms. However, these types of heterologous expression require for full desaturase functionality the presence of a suitable membrane environment and electron donor (cytochrome $b_5$). In addition, homologous transferring of desaturase genes from one microbial species into others offers much potential for exploitation to develop strains with the desired PUFAs (49). Thus, these functional exchanges of desaturase genes from different origins are important steps towards the production of special PUFAs in oleaginous microorganisms.

**Desaturase inhibitors** Another strategy in modifying

PUFA profile is based on the employment of desaturase inhibitors that control individual desaturation steps. Probably the first known naturally occurring inhibitor of unsaturated fatty acid desaturation is cyclopropene sterulic acid which specifically blocks the conversion of stearic acid to oleic acid catalyzed by $\Delta^9$ desaturase. Sterulic acid was efficiently used for production of microbial oils having fatty acid composition similar to cocoa butter (50). Various other cyclopropene fatty acid esters have been synthesized as a potential structure-based inhibitors of $\Delta^6$, $\Delta^{12}$ and $\Delta^{15}$ desaturases (51).

Three other inhibitor types showing unique effects on PUFA metabolism were discovered during studies on $C_{20}$ polyunsaturated fatty acid biosynthesis in *Mortierella* fungi. The first type consists of lignan compounds isolated from the nonoil fraction of sesame oil (52) and from Chinese medicine "Saishin" (*Asiasari radix) (53).* The sesamin analogues specifically and noncompetitively inhibit $\Delta^5$ desaturase with $K_i$ values in the range of 160–710 $\mu$M. None of the $\Delta^6$, $\Delta^9$, and $\Delta^{12}$ desaturases were inhibited by low concentrations of these lignans. The second type involves alkyl gallate antioxidants which noncompetitively inhibit both $\Delta^5$ and $\Delta^6$ desaturases with $K_i$ values of 26 $\mu$M and 170 $\mu$M, respectively (54). The inhibitory function requires special structural features such as the aromatic ring carries a hydroxyl group at the metha position and the carboxyl group esterified with an alcohol of appropriate carbon length. The third type composes of curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a major component of turmeric (55, 56). It has a noncompetitive inhibitory effect on both $\Delta^5$ ($K_i$ = 36 $\mu$M) and $\Delta^6$ desaturase ($K_i$ = *28* $\mu$M). Although curcumin has a symmetrical structure with a methylene group as the center, only half of the structure is essential for the desaturase inhibition.

Several other unnatural desaturase inhibitors have been synthesized. For example, anisole derivatives block $\Delta^5$ desaturase in fungi (57). Some analogues of fatty acids, *trans*-octadecenoic acids, were found to be competitive inhibitors of $\Delta^9$, $\Delta^6$, and $\Delta^5$ desaturases in rat liver microsomes. Herbicides of the substituted pyridazinone family showed inhibitory effects against $\Delta^6$ and $\omega^3$ desaturases in microalgae (58). On the other hand, Ca-channel blockers nicardipine and nifedipine inhibit rat microsomal desaturases noncompetitively (59). While nicardipine specifically reduces $\Delta^5$ desaturase ($K_i$=62 $\mu$M), nifedipine efficiently blocks $\Delta^6$ desaturase ($K_i$=44 $\mu$M). Interestingly, when these two compounds were applied to fungal desaturases, no inhibition was observed. Conversely, verapamil, another Ca-channel blocker, reduces both microbial $\Delta^5$ and $\Delta^6$ desaturases, while no effect on the liver microsomal desaturases was observed. The reason for these discrepancies may be derived from the different properties of the fungal and human desaturases. Thus, the application of desaturase inhibitors to biochemical and genetical studies could be a valuable tool for elucidation of desaturation complexes from various sources.

**Enzymatic biotransformations** The application of lipase-catalysed reactions in many industrial fields has led to incorporation of these technologies to the improvement of lipid composition from various sources. Employment of enzymatic routes to obtain desired PUFA oils is an attractive alternative to chemical processes because (i) formation of undesirable coproducts is almost eliminated under the mild conditions characteristic for enzymatic conversions, and (ii) the PUFA profile of the product can be engineered by appropriate selection of biocatalysts and reaction conditions (60). In particular, the enzymatic interesterifications are useful for modifying microbial TAGs, where their nutritional values are based not only on the degree of unsaturation, but also on the acyl chain length and positional composition of individual PUFAs in the glycerol backbone (61-63). Therefore, these tailor-made TAGs will make desirable compounds for nutritional supplements, infant formulas and therapeutic agents because of their superior assimilation and metabolism. In addition, sufficient lipase-assisted incorporation of w-3 PUFAs to the oils containing $\omega$-6 PUFAs allows the preparation of oils with desirable $\omega$-6/$\omega$-3 PUFA ratios, leading to more beneficial human applications (64, 65).

The commercial practicability of lipase-mediated bioconversions has been clearly demonstrated by producing highly purified PUFAs, unique TAGs, PUFA-based bioemulsifiers and phospholipids for use in foods, nutraceuticals, biomedicals, pharmaceuticals, cosmetics and drug-delivery devices (66). In addition, lipase-based processing can potentially provide access to bulk and refined PUFA feedstock suitable for further biological applications that have been hindered by the high cost of PUFAs refined by current chemical procedures. Finally, microbial PUFAs could be incorporated into seed and animal oils providing cheap PUFA-fortified oils suitable for direct use.

## MICROBIAL PRODUCTION OF PUFAs

Increasing demand for high-value lipids has focused commercial attention on the provision of suitable biosynthetic framework for their production. Particularly active in the synthesis of these and related fatty acids are species of fungi belonging to Zygomycetes (10). Oleaginous fungi producing PUFA could be economically valuable providing that the most of their PUFAs occur in the triacylglycerol fraction of their lipids (Table 2). Microalgae are also potential sources of these fatty acids, where production of w-3 PUFAs from microalgae is being reconsidered and is expected to proceed rapidly (67). However, they require special growth conditions and harvesting/recovery techniques, so their use is not always economically feasible. The possibility that a stable economic niche exists for microbial PUFAs has led a number of companies to subsidize development of these biotechnological methods. For example, Rhône-Poulenc, Gist Brocades, Suntory, Idemitsu, Martek and Lion have developed processes for fungal PUFAs. Microalgal approaches to form PUFAs have been progressed by Martek, Nisshin Oil Mills, Kelco International, Omega-Tech, Scotia Pharmaceutical and Heliosynthese. On the other hand, Sagami and Nestle have examined bacterial PUFA-rich oils. The competition among fungi, microalgae, bacteria and plants as sources of PUFA will undoubtedly serve as a stimulus for all concerns, and it will be of considerable interest to see which routes eventually emerge as the economically preferred ones for the production of individual fatty acids.

**$\gamma$-Linolenic acid** GLA was the first microbial PUFA studied extensively. Although a number of filamentous fungi of the class Zygomycetes accumulate large amounts of oil, they tend to have a low content of GLA and, conversely, those with high GLA content have only

TABLE 2. Selected microorganisms for polyunsaturated fatty acid production

| PUFA | Strain | TL/DCW[a] (%) | PUFA formation[b] (% in oil) | (% in DCW) | (g/l) | Remark | Ref. |
|---|---|---|---|---|---|---|---|
| GLA | *Mortierella ramanniana* | 50 | 17.6 | 8.9 | 5.5 | | 70 |
| | *Mortierella isabellina* | 53 | 4.5 | 2.2 | 3.4 | | 69 |
| | *Mucor circinelloides* | 20-25 | 15-18 | ND[c] | ND' | | 68 |
| | *Mucor circinelloides* | 23 | 24.3 | 5.5 | 0.8 | | 57 |
| DGLA | *Conidiobolus nanodes* | 34 | 18.3 | 6.2 | 1.6 | $\Delta^5$ inhibitor add. | 57 |
| | *Mortierella alpina* | 45 | 26.8 | 10.7 | 2.6 | $\Delta^5$ inhibitor add. | 86 |
| | *Mortierella alpina* | 50 | 42 | 20.5 | 4.1 | $\Delta^5$ mut | 88 |
| AA | *Mortierella alpina* | 45 | 50 | 22 | 13.0 | | Ref[d] |
| | *Mortierella alpina* | 44 | 46 | 23 | 11.1 | 15°C | 9 |
| ETA | *Mortierella alpina* | 30 | 26 | 7.7 | 1.6 | $\Delta^5$ mut, ALA add. | 108 |
| | *Mortierella alpina* | 26 | 37 | 9.7 | 2.3 | $\Delta^{12+5}$ mut, ALA add. | Ref[e] |
| EPA | *Mortierella alpina* | 55 | 12 | 6.7 | 1.9 | low temp, ALA add. | 94 |
| | *Mortierella alpina* | 32 | 20 | 6.4 | 1.0 | $\Delta^{12}$ mut, ALA add. | 95 |
| | *Mortierella elongata* | ND' | 15.1 | ND[c] | 0.6 | | 91 |
| | *Pythium ultimum* | 18 | 20 | 3.4 | 0.4 | | 97 |
| DPA-$\omega$6 | *Schyzotrichium* SR21 | 53 | 7.4 | 4.8 | 1.0 | | 106 |
| DHA | *Schyzotrichium* SR21 | 70 | 37.3 | 26.2 | 15.5 | | 105 |
| | *Crypthecodinium cohnii* | 60 | 39 | 25.0 | 8.0 | | Ref[f] |
| | *Schyzotrichium* SR21 | 53 | 34.0 | 22.4 | 4.7 | | 106 |
| | *Thraustochytrium roseum* | 23 | 48.8 | 11.7 | 2.0 | | 103 |
| 18 : 2 $\omega$9 | *Mortierella alpina* | 40 | 14 | 6.0 | 1.0 | $\Delta^{12}$ mut | 110 |
| | *Mortierella alpina* | 40 | 16 | 6.3 | 1.1 | $\Delta^{12+5}$ mut | 111 |
| 20 : 2 $\omega$9 | *Mortierella alpina* | 44 | 25 | 11.0 | 1.7 | $\Delta^{12+5}$ mut | 111 |
| MA | *Mortierella alpina* | 43 | 33 | 14.1 | 1.9 | $\Delta^{12}$ mut | 110 |

[a] Total lipid per dry cell weight (%, w/w).
[b] PUFA concentration in oil and dry biomass (DCW) is expressed as %, w/w.
[c] Not disclosed.
[d] Higashiyama, K. *et al.,* Abstr. AOCS Annu. Meet., Chicago, p. 34, 1998.
[e] Certik, M. *et al.,* Abstr. 10th Plant Lipid Symp., Japan, p. 6, 1997.
[f] Kashiwakura, M. *et al.,* Proc. 3rd Internatl. Marine Biotechnol. Conf., Norway, p. 61, 1994.

low oil levels. The most suitable species appear to be molds of the order ***Mucorales.*** Some of them give a satisfactory overall GLA content in biomass (about 4%) that ensures a relatively high oil yield (more than 20% in biomass) with appropriate concentration of GLA (20–25%). Another practical source of GLA is microalgae ***Spirulina platensis,*** which has been marketed as a health food in several countries, but its effectivity to form GLA is not as high as Zygomycetes (58).

The first commercial-scale microbial process was developed in 1985 in the United Kingdom with ***Mucor circinelloides (M. javanicus).*** In a 200 kl fermentor the strain accumulated 20% lipid in the biomass and the oil contained 15–18% GLA (68). Although the oil after extraction and refining consisted of 98% TAG, and the GLA content was twice that of evening primrose oil, marketing difficulties were the principal reason for suspension of this industrial production. The other biotechnological process (two-stage continuous fermentor system) for obtaining GLA-rich oil is still continuing in Japan using strains of ***Mortierella*** and ***Mucor.*** The cultivation of ***Mortierella isabellina*** leads to 83 g lipid/l (4.5% GLA in total fatty acids) representing 3.4 g GLA/*l* (69). On the other hand, a new mutant of *Mortierella* ***ramanniana,*** grown in a fermentor equipped with a special mixing system for highly viscous media, improved GLA production to 5.5 ***g/l*** (more than 30g lipid/l with 18% GLA in oil) (70). This oil is commercially used as a food additive or as a health food in the form of drinks, candy, jelly and the oil is also added to cosmetic preparations.

Another attempt to commercialize fungal GLA biosynthesis has been developed in Slovakia, and the strains of ***Mucor mucedo*** and ***Cunninghamella echinulata*** forming up to 30 mg GLA/g mycelia were considered as perspective GLA producers (17). Attention is now focused on the isolation, purification and use of the oil as a dietary supplement for human and animal applications. Efforts to industrialize microbial GLA biosynthesis have also been performed by Kennedy ***et al.*** (29), who constructed a fungal model for the maximal GLA production and applied it to large-scale fermentations. To reduce cost, the relationship between monocarboxylic acids (as waste materials from petrochemical processes) and GLA formation in fungi has been studied in South Africa (71). Another strategy based on the use of ***Mucor ambiguus*** immobilized on porous support particles enhanced GLA productivity and at the presence of nonionic surfactants, lipid containing GLA was found on the cell wall surface and excreted into the culture broth (72). Alternative methods of fungal fermentation with simultaneous production of other useful metabolite(s) have also been proposed with the aim of decreasing the price of microbial GLA-oils. For example, ***Mucor circinelloides*** was found to produce both GLA and cocoa butter equivalents in substantial amounts (73). Strain ***Rhizopus arrhizus*** showed high saccharide conversion to extracellular L(+)-lactic acid and the biomass served as an advantageous source of GLA (74).

SSF processes using various types of cereals as nutrient sources have been successfully employed for GLA production. Generally, the surfaces of substrates were covered by the fungal mycelium after l-3 d of cultivation and total yield of prefermented mass decreased during cultivation. ***Mortierella isabellina*** CCM-14 was found to be a promising GLA producer (Slugen, D. ***et***

*al.,* Czech Patent 279043, 1994). Its cultivation on barley led in 18.6% GLA in total oil, that corresponds to 18 mg GLA/g bioproduct (prefermented residual substrate with fungal biomass). High production of GLA by ***Cunninghamella japonica*** was also reported where the fungal growth on rice and millet resulted in 7.0–7.9% GLA in bioproduct (20% GLA in total oil) (75).

**Arachidonic acid** The ability to accumulate AA at an industrial level is observed mainly in the ***Mortierella*** genus (13, 14), although other strains were also studied (76). AA production by fungi can be substantially enhanced by altering the cultivation conditions where AA concentration in the oil varies between 30-70% with 70-90% of the AA formed being bound to TAG (9, 37, 77). The proportion of AA in the fungal oil was almost doubled (up to 70%) when harvested mycelia are stored for 6 d at laboratory temperature (78) or at low temperature (79, 80). As the temperature decreased, AA concentration was elevated in phospholipids as a result of the adaptation mechanisms of the fungi to maintain membrane fluidity. Manganese ions promoted AA formation in ***Mortierella alpina,*** whereas iron ions at concentrations above 40 mg/*l* strongly inhibited AA accumulation (81). Efforts to increase AA formation by computerized optimization of several variables affecting AA synthesis in ***Mortierella alpina*** were performed (82). The highest yield of 13 g AA/l (220 mg/g mycelia) was achieved by ***Mortierella alpina*** 1S-4 in a 10 kl fermentor (Higashiyama, K. ***et al.,*** Abstr. AOCS Annu. Meet., Chicago, p. 34, 1998). This fungal process is evidently superior to microalgal processes among which ***Porphyridium cruenturn*** is probably the best producer of AA (58).

Solid-state fermentations were also employed to improve the market for fungal AA-rich products. From the screening of many fungi, ***Mortierella alpina*** CCF-185 showed highest AA productivity (83), where 57.4 mg AA/g bioproduct (49% AA in oil) was achieved after the strain was grown on millet. Totani ***et al.*** (84) reported production of 11.8 mg AA/g potato/dextrose paste by ***Mortierella alpina.***

**Dihomo-$\gamma$-linolenic acid** Growth of most fungi normally forming AA leads also to production of small amounts of DGLA. Strains with moderate AA contents were able to form higher concentrations of DGLA and also GLA when cultivated on a potato-glucose agar medium containing acetylsalicylic acid (14). Because AA is synthesized from DGLA by $\Delta^5$ desaturase, all AA-producing fungi should potentially produce large amounts of DGLA if this conversion is efficiently blocked. Therefore, two strategies were employed to reach this goal. One is based on the presence of $\Delta^5$ desaturase inhibitors in cultivation medium. Among various substances tested, addition of 3% sesame oil to a medium led to remarkable increase of DGLA amount by *Mortierella* ***alpina*** up to 1.7 ***g/l,*** whereas AA production was only 0.7 ***g/l*** (85). Optimization of the culture conditions with addition of sesamin resulted in 2.6 g DGLA/*l* (107 mg/g mycelia; 27% in oil) (86). Similarly, DGLA production by ***Mortierella alpina*** at the presence of peanut oil reached 1.9 ***g/l*** (87) and the use of tert-butyl hydroxy anisole in the medium yielded 1.6 g DGLA/*l* by ***Conidiobolus*** *nanodes* (57). Other $\Delta^5$ desaturase inhibitors were also reported (see above) but their effectivity in stimulation of DGLA production is not as high as with sesamin. The second approach is the use of $\Delta^5$ desaturase-defective mutants of ***Mortierella alpina*** which are characterized by a high DGLA yield (4.1 ***g/l;*** 42% in oil) and a reduced concentration of AA (88). Production of DGLA by these mutants is advantageous because it does not require inhibitors and its yield is relatively high.

**Eicosapentaenoic acid** Most of the AA-producing fungi also form detectable amounts of EPA and its concentration in the oil increases when fungi (especially ***Mortierella*** spp.) are cultivated at low temperature. The enzyme system of ***Mortierella*** spp. catalyzing the conversion of AA to EPA ($\Delta^{17}$ desaturase) is activated by cold (89) and the resultant EPA is necessary to maintain the proper membrane fluidity at low temperature (90-92). The best EPA yield (0.6 g/I; 15.1% in oil) was obtained with ***Mortierella elongata*** (91). Accumulation of EPA was improved by addition of ALA to the media and under these conditions ***Mortierella alpina*** formed 1.35 g EPA/l (93). Moreover, conversion of ALA to EPA is temperature independent which markedly reduces the energy cost for the fermentation than lowering cultivation temperature. However, simultaneous operation of temperature-dependent conversion of AA to EPA and ALA addition to media at low temperature further stimulated EPA formation to yield of 1.9 g EPA/l (94).

SSF process has also been developed to prepare EPA-rich bioproducts using fungi that can efficiently utilize, incorporate and modify exogenously added oils in their cells. ***Mortierella alpina*** grown on a mixture of barley and thresh supplemented with linseed oil (consists of about 57% ALA) yielded 23.4 mg EPA/g bioproduct (17.8% EPA in oil) and 36.3 mg AA/g bioproduct (27.6% AA in oil) (Slugen, D. ***et al.,*** Czech Patent 279043, 1994). Subsequent storage of the bioproduct at 10°C for 7 d resulted in the increase in the ratio of the total $\omega$-6/$\omega$-3 fatty acids from 0.9 to 1 .l as well as the ratio of PUFA/saturated fatty acids from 6.0 to 7.8.

Nevertheless, AA concentration in the mycelial oil is usually higher than that of EPA, even under the conditions described above. From a nutritional point of view, an EPA-rich oil with a low AA level is preferable, because of the diverse biological activities of AA (2). Therefore, $\Delta^{12}$ desaturase-defective mutants unable to synthesize any $\omega$-3 or $\omega$-6 PUFAs when grown on glucose was employed to solve this problem (95). Because the other desaturases and elongases are still active, ALA added exogenously (as linseed oil) to the medium was efficiently converted to EPA with concomitant decrease in AA content to achieve the final mycelial EPA/AA ratio of 2.5. Another fungus, ***Saprolegnia*** sp. efficiently utilized some natural oils as carbon source and converted them to EPA (96). Under optimal growth conditions at low temperature with addition of olive oil, EPA/AA ratio reached the value of 10.9 (2.3% EPA in biomass).

Although EPA may constitute up to 25% of the total fatty acids in some species of ***Mortierella*** (91) and *Pythium* (97, 98), microalgae with almost 50% EPA in their oil (58) strongly compete with fungi in industrial EPA production. Kyle's group of Martek Corp. has pioneered the use of this approach for the production of EPA-rich oils (99). The advantage of this approach is the absence of all other PUFAs in microalgal oils making EPA purification relatively easy. Marine bacterium ***Shewanella putrefaciens*** is also considered as another alternative source for commercial EPA production (100, 101). This bacterium accumulates up to 15% of lipid in the cell, of which 25–40% is EPA. However, all of the EPA is

bound to phospholipid membranes causing additional difficulties for EPA recovery.

**Docosahexaenoic and docosapentaenoic acid** DHA occurs in several marine fungi, of which *Thraustochytrium aureum* accumulates up to 50% this fatty acid in oil (102, 103). Unfortunately, the oil content of this mold is only 10–15% and the cells grow slowly with low yield. Moreover, the cell fragility at high content of intracellular PUFAs makes fermentation development difficult (104). On the other hand, Yaguchi *et al.* (105) reported that strain *Schizochytrium* SR21 is tolerant to mechanical stirring of up to 500 rpm using a propeller-shape impeller and after cultural optimization in a fermentor the strain yielded 15.5 g DHA/*l* in 5 d. In addition, the same strain grown under slightly modified conditions yielded both 4.7 g DHA/*l* and 1.0 g DPA-$\omega$6/*l* (106) making the strain SR21 an excellent source of DHA and DPA-$\omega$6. Moreover, high proportion of DHA and DPA-$\omega$6 in the oil and relatively low levels or absence of other PUFAs simplifies downstream processing of these two fatty acids. Other microbial sources of DHA including fungi, microalgae and psychrophilic bacteria were recently reviewed by Singh and Ward (103), but their DHA productivity is not as high as that mentioned above.

An interesting method for obtaining high DHA yield of 8 g/l (39% in oil; 25% in biomass) by heterotrophic semicontinuous fermentation of the marine algae *Crypthecodinium cohnii* and the subsequent use of DHA for aquaculture of marine juvenile fish was reported (Kashiwakura, M. *et al.,* Proc. 3rd Internatl. Marine Biotechnol. Conf., Norway, p. 61, 1994). Experimental nutritional enrichment of shrimp followed by brining for preservation led to 30% DHA in shrimp oil or 3% DHA in dry shrimp body without any loss in DHA biological activity.

**Eicosatetraenoic acid** ETA is another product of interest because of its potential use as a precursor of prostaglandins (107). One method to produce oil containing ETA has been developed using desaturase-defective mutants of *Mortierella alpina.* Mutants defective in $\Delta^5$ desaturase grown in medium supplemented with ALA are able to accumulate oil with 26% ETA (1.6 g ETA/Z) (108). Because these mutants usually synthesize also DGLA, its formation should be limited in order to increase the accumulation and improve the purification of ETA. Therefore, mutants defective in both $\Delta^5$ and $\Delta^{12}$ desaturases when cultivated with exogenously added ALA exhibit increased production of ETA (37% in oil, 2.3 g/l) with simultaneous lowering of DGLA content (Certik, M. *et al.,* Abstr. 10th Plant Lipid Symp., Japan, p. 6, 1997).

**Mead acid** MA is known to be a precursor of $LT_3$ leukotrienes and a reductant of inflammatory diseases (109). Its formation occurs via sequential conversions of oleic acid, consisting of $\Delta^6$-desaturation, elongation and finally $\Delta^5$-desaturation (Fig. 2). Occurrence of this fatty acid is very rare among microorganisms because they usually rapidly convert oleic acid to linoleic acid by $\Delta^{12}$ desaturase and linoleic acid may then be further metabolized to o-6 and o-3 PUFAs. Attempts to produce MA resulted in the isolation of $\Delta^{12}$ desaturase-defective strains of *Mortierella alpina,* which under optimal conditions formed 1.9 g MA/I (33% in oil) (110). On the other hand, eicosadienoic acid (20 : 2 $\omega$9) is accumulated in large quantities (1.7 g/l; 25% in oil) when mutants of *Mortierella alpina* with both blocked $\Delta^5$ and $\Delta^{12}$

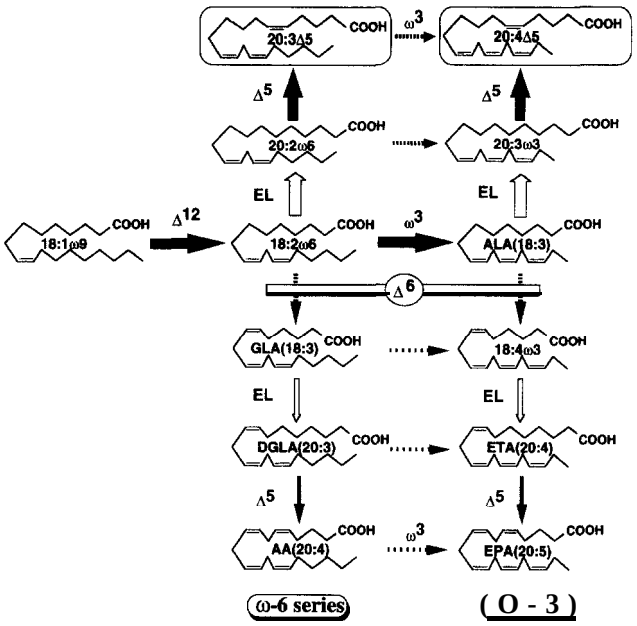


FIG. 3. Biosynthesis of non-methylene-interrupted o-6 eicosatrienoic (20 : 3$\Delta$5) and w-3 eicosatetraenoic (20 : 4$\Delta$5) acids by $\Delta^6$ desaturase-defective mutants of *Mortierella alpina.* EL-elongase; $\Delta^5$, $\Delta^6$, $\Delta^{12}$ and $\omega^3$-desaturases. Defective $\Delta^6$ desaturase and non-methylene-interrupted fatty acids are highlighted.

desaturases are employed (111).

**Unusual polyunsaturated fatty acids** Several fungi have been reported to accumulate unusual PUFAs in their cells. Mycelium of *Mortierella alpina* CBS 219.35 cultivated with glucose contained significant amounts of $C_{19}$ PUFAs (19 : 3$\omega$5 and 19 : 4$\omega$5) together with the common $C_{18}$ and $C_{20}$ PUFAs. On the other hand, *Mortierella alpina* 1S-4 converted 15 or 17 carbon n-alkanes to $C_{19}$ PUFAs efficiently and their accumulation reached over 95% of the total fatty acids (37). Concentration of 19 : 4$\omega$5 acid in the lipid was 11.2% whereas AA was accounted for only 1.5%. Similarly, EPA-producing aquatic filamentous fungus *Saprolegnia* sp. synthesized odd chain PUFAs during growth on medium supplemented with 13, 15 and 17 carbon fatty acids (112). Among them, unusual $C_{19}$ PUFA, 19 : 5$\omega$2 was detected.

*Mortierella alpina* 1S-4 also accumulates a novel o-l eicosapentaenoic acid (20 : 5 *cis* 5,8,11,14,19), w-l eicosatetraenoic acid (20 : 4 *cis* 8,11,14,19), w-l hexadecenoic acid (16 : 1 *cis* 15) and two o-l octadecenoid acids (18 : 1 *cis* 17; 18 : 4 *cis* 6,9,12,17) when grown on a medium with 1-hexadecene or I-octadecene (113). It has been suggested that the $\omega$ methyl group may be first oxidized to a carboxyl group and the resultant o-l fatty acids are further utilized via the w-6 route.

Mutants of *Mortierella alpina* defective in $\Delta^6$ desaturase produce two non-methylene-interrupted PUFAs, w-6 eicosatrienoic (20 : 3$\Delta$5) and w-3 eicosatetraenoic (20 : 4$\Delta$5) acids (Fig. 3) (114). 20 : 3$\Delta$5 is thought to be synthesized by the elongation of linoleic acid and then by $\Delta^5$-desaturation. The formation of 20 : 4$\Delta$5 might be initiated by conversion of linoleic acid to ALA ($\omega^3$-desaturation) followed by elongation and $\Delta^5$-desaturation. Although the biological activities of non-methylene-interrupted fatty acids are not fully understood, they are probably formed in response to a deficiency of o-3 and o-6 PUFAs. 20 : 3$\Delta$5 can be converted via cy-

clooxygenase to hydroxy-fatty acids (115) and may alter eicosanoid signalling (116).

## DOWNSTREAM PROCESSING OF MICROBIAL OILS

**Extraction** The development of microbial lipid production has mainly been concentrated on the organism selection and optimization of cultural conditions. Unfortunately, less attention has been focused on oil isolation and most extraction methods applied to microbial system have been originally described for animal tissues and plant materials. Therefore, reliable processes for recovery and purification of microbial oils have to be employed to further develop this area of microbial biotechnology. The choice of an isolation procedure depends on both the nature of the microbial cells and the type of extract desired. A major problem which causes much troubles is the failure to prevent lipolysis occurring during lipid recovery processing. Satisfactory treatment must also be used to minimize autooxidative degradation and the presence of artifacts. Moreover, if microbial oils are considered to be used for human purposes, solvents must be acceptable in terms of toxicity, handling, safety and cost.

From these points of view, the application of two-step extraction with ethanol and hexane leading to high yield of GLA-rich fungal oil (Suzuki and Yokochi, US Patent 4 870 001, 1989; 117) is convenient and was also recommended for oil isolation from yeasts (118). However, a high degree of lipid contamination was observed with this method in which some enzymes (predominantly lipases) were not inactivated and catalyzed undesirable reactions. As an alternative, Sakaki et ***al.*** (119) investigated supercritical fluid extraction using fungal model, but only under laboratory conditions. Recently, Certik and Horenitzky (120) extended the process of supercritical $CO_2$ extraction of GLA-containing oil from the fungus ***Cunninghamella echinuiata*** to the semi-industrial scale. Resultant GLA-oil had qualities similar to oil obtained by conventional hexane/alcohol or chloroform/methanol methods and its yield was slightly higher. Although hexane extraction is still costly superior to supercritical $CO_2$ extraction, this method could be further improved and used to obtain fungal GLA-containing preparations having commercial applications in the pharmaceutical, medical and nutritional fields.

**Refining and modification** Final processing of isolated microbial oil involves purification or refining, and modification. The refining treatment is necessary to remove or reduce, as much as possible, those contaminants of the crude oil which will adversely affect the quality of the end-product and the efficiency of the modification procedures. Certain compounds in the crude oil, notably the tocopherols and tocotrienols as antioxidants, have a beneficial effect on product quality, and therefore the refining treatment should be designed in a way that as much as possible these compounds are retained in the oil. Purification and concentration of microbial oils require the use of several processes depending on the extracted fatty acid and the operation scale. This includes urea adducts formation, separation on Y-zeolites, solvent winterization, differential crystalization, various chromatographic techniques and lipase-catalyzed reactions. It is not advisable to distil PUFAs or their esters if this can be avoided since they are liable to undergo double-bond migration, strereomutation, cyclization and dimerization under extreme thermal conditions. Where distillation is unavoidable, artefacts must be subsequently removed. The modification procedures include hydrogenation, fractionation and inter-esterification. They are used to extend the applicability of microbial oils and also, in the case of hydrogenation, to make products with acceptable oxidative and flavour stability from relatively unstable raw materials.

Fungal GLA oil, compared with the plant oils (40-70% linoleic acid), is by far the most useful starting material for GLA purification, because the lower content of linoleic acid makes GLA purification easier, although GLA content in fungal oil itself is similar to that of plant oils. The mixture of fatty acid methyl esters from ***Mortierella*** sp. (6.8% GLA) and ethyl esters from ***Mucor circinelloides*** (25.1% GLA) were fractionated with urea to obtain oil with 97.5% GLA at 50–60% recovery (57, 121), further purification by HPLC led to 99.5% GLA (57). Lipase-catalyzed selective esterification with n-butanol was efficiently applied to enrich GLA of fungal unesterified fatty acids from 10.4% to almost 70% (122). In addition, high GLA recovery (90–95%), although originally applied to borage oil, was also obtained by treatment with acyl-specific fungal lipase and yielded fatty acid with GLA content of 70% (123) or 87% (62). On the other hand, GLA ethyl esters of 98% purity was obtained from a mixture of fungal fatty acid esters using various types of Y-zeolites (124).

There have also been attempts to purify other PUFAs. Because individual PUFAs have different properties, concentration steps for each PUFA must be optimized separately. For example, linoleic acid and GLA were removed by distillation prior to purification of fungal DGLA ethyl esters (13.7% DGLA) and subsequent urea treatment yielded 51.5% DGLA (57). Sajbidor ***et al.*** (125) reported that selection of several purification procedures allows a simple preparation of fungal AA with a purity of 78% (urea treatment of fatty acids) to 90% (urea fractionation of methyl esters). Hydrolysis of *Mortierella* sp. oil by yeast lipase increased AA content in the TAG fraction by up to 60% (61) and several hydrolyzation and selective esterification steps of fatty acid fraction resulted in 75% AA (126). For the next DGLA or AA purification step (above 90%), the applications of HPLC (57), column chromatography on octadecylsilane (Shinmen, Y. ***et al.,*** Eur. Patent Appl. 0 276 541,1987) or thin layer chromatography on reverse phase (Totani, N. ***et al.,*** Eur. Patent Appl. 0 223 960,1986) were proposed. The other three-step method that includes separation of glycolipids, formation of a urea inclusion complex and reverse phase chromatography of algae oil resulted in 97% EPA or 80% AA concentrate (127). Urea fractionation of fatty acid methylesters was also beneficial for gaining highly unsaturated fungal oil with 73.3% DHA and 17.7% DPA-$\omega$6 (106). Industrial-scale purification of these two PUFAs was developed on the basis of HPLC techniques, in which fungal DHA and DPA-$\omega$6 ethyl esters were obtained in greater than 99% purity (128). Also, a number methods with the aim of preparing PUFA-rich products from natural sources have been described. Perhaps the simplest process lies in the enzymatic treatment of oils resulting in satisfactory enrichment of both w-6 and w-3 PUFAs in the form of TAG (62-65, 129, 130) or as free fatty acids (62, 123, 131). Moreover, the yield of these acids can be improved

by removal of by-product of lipase-catalyzed interesterification by continuous supercritical $CO_2$ extraction (132).

## CONCLUSION AND FUTURE PERSPECTIVES

Growing interest in PUFA applications in various fields coupled with their significance in health and dietary requirements has encouraged "hunting" for more suitable sources of these compounds. Inadequacy of conventional agricultural and animal oils has put attention on developing new microbial technologies employing certain fungi, microalgae and bacteria. However, the focus of biotechnology on highly valuable PUFAs requires knowledge how microorganisms control and regulate the fatty acid biosynthetic machinery in order to obtain specific PUFA in high yield. Because these microbial processes could only be economically viable when most of the PUFAs occur in the triacylglycerol form, elucidation of the mechanisms and identification of the key steps that limit the flow and incorporation of the desired fatty acid into triacylglycerols may facilitate future progress in this area. Similarly, understanding the structure/function relationship between desaturases, acyltransferases and other proteins involved in the complete assembly of lipogenesis will help to improve microbial oil quality. Comparative successes using mutation methods and molecular engineering techniques carried out over recent years have not only answered some fundamental questions related to fatty acid formation but has also enabled the construction of new microbial varieties that can synthesize unusual fatty acids. Isolated desaturase genes offer much potential to exploit more the transgenic approach in order to create novel PUFAs for the pharmaceutical and nutraceutical industries and open up new possibilities to control microbial oil types assembled. Elucidation of the signalling systems and mechanisms transmitting the signals from different membranes to the major sites of lipid biosynthetic machinery represents a challenging and potentially rewarding subject for the further research and with the amount of knowledge acquired so far will finally allow us to move from empirical technology to predictable oil design. Future prospects of biotechnological applications of oleaginous microorganisms are also related to commercially interesting products including hydroxy-PUFAs, prostaglandins, leukotrienes, thromboxanes or other useful lipid classes, which are extraordinarily expensive to produce by chemical synthesis. Thus, the manipulation and regulation of microbial lipid biosynthesis open a large number of possibilities for academic research and demonstrates the enormous potential in its application.

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