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PRODUCTION OF POLYUNSATURATED FATTY ACIDS BY FUNGI: A REVIEW

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ABSTRACT

Omega (ω)-6 PUFAs, or especially gamma linolenic acid (cis, cis, cis 6,9,12 octadecatrienoic acid) comprised of 18 carbon atoms and three double bonds is also known as gamolenic acid. Gamma Linolenic Acid (GLA) is found naturally in varying extends in the fatty acid fraction of some plant seed oil. Recent research suggests that gamma (γ) -linolenic acid is unique among the n-6 polyunsaturated fatty acid (PUFA) family members as it ameliorates many health problems. There are many evidences suggesting GLA for pharmacological and dietetic purposes. Over the past several years' extensive research has been made for the microbial production of PUFA. The focus has been laid down to maximize PUFA yield by novel mutations, genetic manipulations, isolating new strains as well as optimizing the media for cultivating more efficient strains. The aim of this work was to isolate and screen GLA-producing filamentous fungi and to optimize various process parameters for GLA production through systematic manipulation of the fermentation parameters. The thesis also aims at inhibiting the genes responsible for further elongation of the carbon chain in the biosynthetic pathway of this fungus. The ultimate objective of the study is to develop new edible oil based, value-added products by using an optimal source of omega 6 polyunsaturated fatty acids specifically GLA.

NO KEY WORDS: GAMMA LINOLENIC ACID,POLYUNSATURATED FATTY ACID,FUNGI

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INTRODUCTION

Structural diversity and physiological functions of fatty acids and lipids

Lipids are important constituents of human cells and tissues and have several important functions in metabolism relating to the growth and maintenance of the body. They are water insoluble organic biomolecules that can be extracted from the cells and tissues by non-polar solvents, e.g. chloroform. There are several different families or classes of lipids but all derive their distinctive properties from the hydrocarbon nature of a major portion of their structure. Chemically lipids vary to such a great extent that no structural definition is available (Gurr and Harwood, 1991). Lipids have been classified in several different ways. The most satisfactory classification is based on their backbone structures. The complex lipids can be hydrolyzed to yield smaller molecules, but not the simple lipids (McMurry, 1988). The major components in lipids are triacylglycerol (TAG) molecules. The TAG molecules consist of three fatty acids attached to a glycerol backbone; their compositions can vary both within and between organisms. Natural oils and fats consist mainly of triacylglycerols. For this reason, the terms oil and fat are often used to denote triacylglycerols. A fat is a lipid material that is solid at the room temperature, whereas oil is similar material (Staufer, 1996) that is liquid at room temperature (Table 1).

Table 1
Classification of lipids

Lipid Type	Backbone
Complex (saponifiable)	
Acylglycerols	Glycerol
Phosphoglycerides	Glycerol 3 Phosphate
Sphingolipids	Sphingosine
Waxe	Nonpolar alcohols of high molecular weight.
Simple (nonsaponifable)	
Terpenes	
Steroids	
Prostaglandin	

Phosphoglycerols consist of a glycerol backbone esterified to two fatty acids and a phosphate group. Phosphoglycerols are very diverse as in addition to being linked to the glycerol backbone; the phosphate group can be attached to a great variety (Table 2) of groups (Gurr and Harwood, 1991). Fatty acids are composed of a long hydrocarbon chain and a terminal carboxylate group. A great variety of fatty acids exist in nature. Most fatty acids are unbranched and consist of an even number of carbon atoms (Table 3). Fatty acids can be broadly categorized into three types: (i) saturated, (ii) monosaturated and (iii) polyunsaturated fatty acids. The configuration of most double bonds in naturally occurring unsaturated fatty acids is cis (Gunstone, 1992).

Biological functions of lipids

Lipids are important structural components of membranes, which play crucial role in carbon and free energy storage. Lipids are accumulated as lipid bodies in almost all eukaryotic organisms at some point during their life cycle (Murphy, 1991). Evolution has selected triacylglycerols as one of the major storage materials of cells (Stryer, 1988). In times of plenty, triacylglycerols can be stored and used during starvation or strong exercise. Mammals store triacylglycerols mainly in adipose tissue and can excrete them via milk as an energy source for newborn individuals. In plants, triacylglycerols can be stored in the seeds as energy reserves for the germination process. The microorganisms can store triacylglycerol as intracellular oil droplets. The acyl glycerols play no or a little part in membrane structure.

Table 2
The types, structure and functions of lipids

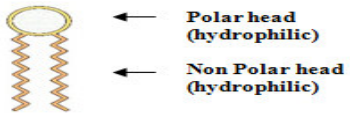
Type	Structure	Function
Triglycerides	Made out of one glycerol molecule with three fatty acids attached These are entirely hydrophobic. The "Tri" prefix of the name comes from having three fatty acids	Those are the "Fats" and also the "oils". They are used for long term. Sugar storage or for insulation of a multicellular organisms.
Phospholipids	Phospholipids have the special property of having both hydrophobic and hydrophilic parts, although they are still mostly hydrophobic. A module that is both hydrophilic and hydrophobic is called amphipathic 	Phospholipids help to make up the basis of cellular membranes.
Steroids (a sterols derivative)	They are entirely hydrophobic and have their own special structure that does NOT include fatty acids. These molecules are all ranged structures, with 4 carbons rings.	This group includes cholesterol, estrogen, testosterone, progesterone and cortisone. Most steroids are used as hormones.
Waxes	These include fatty acids and are hydrophobic	Used for the protective cuticle of plants. (Also helping to prevent evaporation of water from plants). Also used to block our ear canal to any would be a visitor, and other animal use them for protection and lubrication.

Table 3
Some naturally occurring fatty acids

Common name	Systematic name	Short name
Saturated Fatty Acids		
Lauric Acid	Dodecanoic acid	12:0
Myristic acid	Tetradecanoic acid	14:0
Palmitic acid	Hexadecanoic acid	16:0
Stearic acid	Octadecanoic acid	18:0
Monounsaturated Fatty Acids		
Palmitoleic acid	Δ 9-Hexadecenoic acid	Δ 9 16:1
Oleic acid	Δ 9 Octadecenoic acid	Δ 9 18:1
ω-6 Polyunsaturated fatty acids		
Linoleic acid	Δ 9,Δ 12 octadecadienoic acid	ω-6 18:2
γ Linolenic acid	Δ6,Δ9,Δ12 octadecatrienoic acid	ω-6 18:3
Arachidonic acid	Δ5,Δ8,Δ11,Δ14 Eicosatetraenoic acid	ω-6 20:4
ω-3 Polyunsaturated Fatty acids		
α Linolenic acid	Δ9,Δ12,Δ15 octadecatrienoic acid	ω-3 18:3
Eicosapentaenoic acid	Δ5,Δ8,Δ11,Δ14,Δ17-Eicosapentaenoic acid	ω-3 20:5
Docosahexaenoic acid	Δ4,Δ7,Δ10,Δ13,Δ16,Δ19-Docosahexaenoic acid	ω-3 22:6

All double bonds are in cis configuration.

The lipids that contribute to the structure and function of biological membranes are called structural lipids. Structural lipids contain a (long) hydrophobic and a (shorter) hydrophilic part. They can form sheet like double layers.

In addition to lipid bilayers, biological membranes contain about 50% proteins by weight. Together, structural lipids and membrane proteins form the boundaries of all

living cells and intracellular organelles (Gurr and Harwood, 1991).

Polyunsaturated fatty acids

In humans, polyunsaturated fatty acids are not synthesized in sufficient amounts within the body; therefore, they must be obtained through external sources. For this reason they are often referred to as Essential Fatty Acids (Dyal, 2005). PUFAs are essential structural components of phospholipids in cell membranes, where they affect membrane characteristics and functions, such as fluidity, electrolyte transport and hormonal and immunological activities. There is increasing evidence that PUFAs is beneficial for (long term) human health. They may reduce or inhibit risk factors involved in various diseases like cardiovascular diseases (Kang and Leaf, 1996; Kromann and Green, 1980) and inflammatory and immune disorders (Kremer, 1996). Although, the optimal intake of PUFAs has not yet been established, there is some consensus that the PUFA intake should be at least 3% and preferably 8-23% of the total lipid intake (Gill and Valivety, 1997). The British Nutrition Foundation recommended a ω-6 to ω-3 PUFA ratio between 5:1 and 3:1 (British Nutrition Foundation, 1992). The polyunsaturated fatty acids have attracted great interest due to their biological activities, such as lowering of plasma cholesterol level, prevention of thrombosis and also used as wonderful mediators in the biosynthesis of eicosanoids like thrombozanes, prostaglandin and Leukotrienes (Sprecher, 1981). The eicosanoids are molecules that are active in regulation of critical biological functions by altering cell activities. The eicosanoids are biologically active in vertically every mammalian tissue (Stanley and Miller, 1998). Research in the 1980s began to find the role of prostaglandins in many biological processes.

ω-6 PUFAs, or especially gamma linolenic acid (cis, cis, cis 6,9,12 octadecatrienoic acid) is comprised of 18 carbon atoms and three double bonds is also known as gamolenic acid. The Gamma Linolenic Acid (GLA) is found naturally to varying extents in the fatty acid fraction of some plant seed oil. In evening primrose seed oil, it is present in the concentration of 7 to 14% of total fatty acids; in borage seed oil 20 to 27%; and in black currant seed oil 15 to 20%. The GLA is found in some fungal sources (upto 10-20%) depending upon the species (Mahajan and Kamat, 1988). It is produced naturally in the body as the delta 6- desaturase metabolite of the essential fatty acid (linoleic acid). Under certain conditions (decreased activity of delta 6-desaturase enzymes), GLA may become a conditionally essential fatty acid, which is present naturally in the form of triacylglycerols. The stereospecificity of GLA varies among different oil sources. It is concentrated in the sn-3 position of evening primrose and black currant seed oil and sn-2 position in borage seed oil. The GLA is concentrated evenly in both sn-2 and sn-3 position of fungal oil. It is an important intermediate in the biosynthesis of biologically active prostaglandins from linolenic acid, a potent essential polyunsaturated fatty acid. It was soon realized that GLA could be beneficial in treating human diseases. The GLA has been reported to be effective for the prevention and curing of cardiovascular diseases, hypercholesterolemia, menstrual disorders, certain types of cancers, diabetes, allergies, weight loss, and high blood pressure for application in curing certain skin related as well as a variety of other diseases. For the normal human metabolism, the content of GLA in plasma should be about 25 mgL^{-1}. It is also reported to play a role in the improvement of AIDS conditions, for healthy, smooth and glowing skin by increasing moisture-retaining capacities. γ Linolenic acid is unique among the n-6 polyunsaturated fatty acid (PUFA) family members as it ameliorates many health problems (Fan, 1998). GLA administered orally is capable of suppressing human T cell proliferation (Rossetti, 1997). It suppresses activation of T cell (Vassilopoulos, 1997), effective against rheumatoid arthritis (Deluca, 1995) decreases cardiovascular risk factors (Kernoff, 1977) normalizes nerve conduction velocity and sciatic endoneural blood flow (Dines, 1995). GLA may have anti inflammatory and antithrombotic actions. The

anti-inflammatory and anti aggregatory actions may be accounted for by reviewing its role in eicosanoid biosynthesis. These eicosanoids have anti-thrombogenic, anti-inflammatory and anti-atherogenic properties. The PGE-1 inhibits platelet aggregation and has a vasodilation action. The incorporation of GLA and its metabolites in cell membranes may also play a role in the possible anti-inflammatory and anti-proliferative activities. GLA laden triacylglycerols following ingestion undergo hydrolysis via lipases to form monoglycerides and free fatty acids. Once formed the monoglycerides and free fatty acids, are absorbed by the enterocytes. In the enterocytes a reacylation takes place-reforming TAGs that are then assembled with phospholipids, cholesterol and apoproteins into chylomicrons. The chylomicrons are released into the lymphatics from where they are transported to the systemic circulation. In the circulation, the chylomicrons are degraded by lipoprotein lipases and the fatty acids, including GLA. They are distributed to various tissues in the body. Pregnant women and nursing mothers should not use GLA unless recommended by a physician because of the possible antithrombotic activity; those who take hemophiliacs should exercise caution in its use. The GLA should not be used before surgery. There have been no reports of serious adverse events in those taking GLA supplements. It is usually tolerated very well without any significant adverse effects. No interactions between GLA and aspirin, and other NSAID or herbs, such as *Allium sativum* (garlic) or *Ginkgo biloba* (Ginkgo) have been reported. Such interactions if they were to occur might be manifested by nosebleeds and/or increased susceptibility to bruising. If this does occur, GLA intake should be lowered or stopped. GLA is metabolized to the 20 carbon polyunsaturated fatty acid, dihomo gamma linolenic acid (DHLA) or eicosatrienoic acid (ETA) which is converted to prostaglandin E-1 (PGE-1). This is also metabolized to eicosapentaenoic acid (EPA). GLA and DGLA are present as components of phospholipids, neutral lipids and cholesterol esters, mainly in cell membranes and not in the Free State. The PGE-1 is metabolized to smaller prostaglandin remnants that are primarily polar dicarboxylic acids, which most of them are excreted in the urine.

Source of essential fatty acids

The primary sources of EFA's are plants on land and in the sea. Currently the main industrial sources of PUFA's are fish oil, although alternate source are also used, algae, yeast (*S.cerevisiae*), and evening primrose oil (Table 4).

Microbial production of PUFAs

Oleaginous microorganisms, as alternatives to agricultural and animal oil products, have been intensively studied. Certain fungi, bacteria, heterotrophic and phototrophic micro algae, protozoa and mosses contain various PUFAs. The GLA was the first microbial PUFA studied extensively. The occurrence of GLA was first reported in filamentous fungi by Bernhard *et al.,* (1957). Phycomycetes are characterized in their ability to synthesize GLA, whereas the members of ascomycetes and basidiomycetes with a few exceptions produce (linolenic acid (Shaw *et al*., 1966). Most of these investigations were carried out to determine the fatty acid composition of different fungi and to compare them with other groups of organism for their phylogenetic relationships. Evidence is available, which suggests specific functions for (linolenic acids in algae and gamma linolenic acid in protozoa (Erwin and Baoch, 1963). However, no role for either isomer in fungi has been demonstrated. It is suggested that gamma linolenic acid may be vestigial in phycomycetes, relating these fungi phylogenetically to protozoa.

Table 4
Sources of some essential fatty acids.

EFAs	Source
α-Linolenic acid	Green leaves of plants, including Phytoplaktons and algae. Seeds, nuts, legumes (Flax, canola, walnuts and soy)
Linoleic acid	Seeds, nuts, grains and legumes.
Arachidonic acid (AA)	Directly from animal food (Meat, poultry)
Docoshexaenoic acid (DHA)	Animal food, fish

Among algae, *Spirullina platensis* has been studied extensively as a GLA producer (Mahajan and Kamat, 1988). In oleaginous fungi most suitable species appear to be molds of order Mucorales within the class Zygomycetes; phylum Zygomycota.This order includes the genera *Mortierella*, *Rhizopus* and *Mucor*. Generally fungi within this order are best known as saprophytes favor simple sugars than complex molecules. Fungal sources are gaining popularity as potential GLA producers because the productivity from the seed oil is low; also both huge area and long period are required for harvesting seeds. Some suitable strains proposed for GLA production are *Mortierella rammanniana*, *Cunninghamella japonica*, *Entomophthora exitalis, Mortierella isabellina, Mortierella vinacea, Mucor circinelloides*, *Mucor racemosus* and *R.oryzae* (Table 5).

Table 5
Fatty acid profile of Mortierella sp. (Kendrick and Ratledge, 1992)

Cultures	Palmitic	Stearic	Oleic	Linoleic	Gamma Linolenic	Arachidonic
M.alpina	6.6-20.3	4.0-9.9	6.9-30.5	4.9-30.5	3.8-10.9	20.9-69.7
M.hyaline	17.3-22.5	6.7-12.9	29.3-36.5	7.4-9.6	7.3-7.5	11.3-14.4
M.elongata	15.4-19.0	8.3-14.0	30.3-33.3	6.7-7.4	6.0-7.4	14.3-22.8
M.rammanniana	16.0-25.2	1.7-4.3	21.4-41.7	16.1-25.0	13.2-31.4	
M.isabillina	15.6-21.4	1.7-4.3	38.3-51.9	11.6-17.9	6.9-22.9	
M.vinacea	18.1	2.2	46.3	11.4	22.0	

The diversity of microbial species can facilitate the selection of strains producing oil with predominant fatty acids. Although, the contribution of microbial lipids to the oil industry is nearly negligible, however, there are several reasons that favor further research in this field.

- Active lipid synthesizing apparatus makes oleaginous microorganisms perspective oil sources.
- Microbial PUFAs are the higher value oil types rather than resembling the bulk of low priced commodities (soybean oil, palm oil and sunflower oil).
- Their extremely high growth rates on wide varieties of substrates allow utilizing cheap or zeroing cost materials.
- Oil production can be carried out throughout the year, as there is no seasonal or climatic dependence.
- Microbial sources can supply more concentrated pharmaceutical grade PUFAs than other sources with controlled quality.
- Microbial competence to carry out numerous transformation reactions enables the upgrading of PUFA structures and allows the simultaneous formation of lipid and other products.
- The existence of numerous mutants defective in specific enzymes improves production of tailor made oils.
- Oleaginous microbes can be considered as the appropriate hosts into which foreign genes could be cloned for the production of

described fatty acids not found in other microorganisms but which occur in other fat and oil sources.

- Microorganisms provide useful models for studying lipid biochemistry, metabolic and biochemical questions faster than in complex multicellular systems of other organisms.
- Due to the simplicity of microbial metabolic regulation, they can be readily grown under controlled conditions with nutritional regimes that may stimulate or repress the key step of fatty acid formation and allow the manipulation of the lipid yield profile.
- Microorganisms are rich in protein, trace elements, vitamins, antioxidants etc; they can be employed as micro or macronutrients. In general, the metabolism of omega 6 fatty acids that facilitates the prevention and treatment of diseases and disorders has been addressed around eicosanoids changes in the circulatory system. The eicosanoids from different acids are different both structurally and functionally and are sometimes even antagonistic in their effects (Figure 1). In order to achieve lipid accumulation in microorganism, the growth medium should have an excess of carbon substrate and a limiting amount of nitrogen Thus, when the organism grows, it quickly exhausts the supply of nitrogen but it continues to assimilate the carbon source (usually glucose or an alternative carbohydrate). This is then channeled directly into lipid synthesis with the resulting build up of triacylglycerols within the cell as discrete oil droplets. Oil accumulation may reach over 70% of the cell biomass but not in every oleaginous species. Non-oleaginous microorganisms do not accumulate lipid,

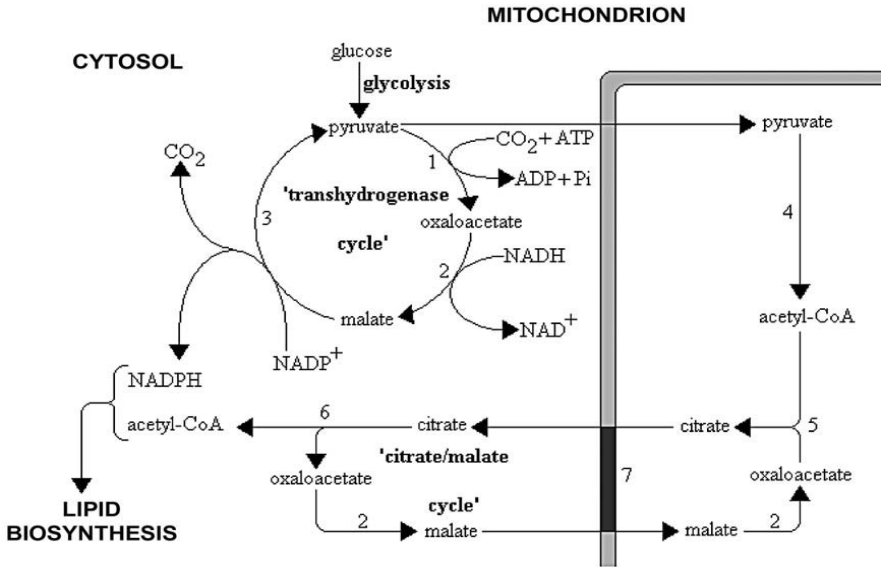


Figure 1
A schematic representation for lipogenesis in oleaginous microorganisms (Ratledge, 2002)

When placed in the same nitrogen limiting growth medium they either tend to cease further cell proliferation or continue to assimilate the available carbohydrate substrate. This is diverted into various polysaccharides, including glycogen and various glucans, mannas, etc. Oil accumulation, beyond a very small level (usually less than 10% of the biomass), does not occur. Therefore, it can be concluded that the ability of an organism to accumulate large quantities of oil must lie outside the immediate area of fatty acid biosynthesis, as this biosynthetic machinery is common to all microorganisms. The reasons for oleaginicity would appear to be two folds:

• The ability to produce a continuous supply of acetyl-CoA directly in the cytosol of the cell as a necessary precursor for fatty acid synthetase (FAS), and
• The ability to produce a sufficient supply of NADPH as the essential reductant used in fatty acid biosynthesis.
The formation of acetyl-CoA in oleaginous microorganisms has been attributed to the presence of ATP: citrate lyase (ACL, reaction no. 1), which does not appear to occur in the majority of non-oleaginous species:
Citrate + CoA + ATP → acetyl - CoA, oxaloacetate + ADP + *Pi*
To operate efficiently, its substrate, citric acid must be made readily available in the cytosol of the cell where fatty acid synthesis occurs.
Citric acid is synthesized as part of the tricarboxylic acid (TCA) cycle within the mitochondrion of the cell. This is a unique feature of oleaginous microorganisms that allows citric acid accumulation so that the activity of isocitrate dehydrogenase as a component of the TCA cycle is dependent on the presence of AMP; no such dependency occurs with the enzyme from non-oleaginous microorganisms. The concentration of AMP itself is regulated by the activity of AMP deaminase (reaction no. 2):
AMP → inosine 5' - monophosphate +NH3
The activity of isocitrate dehydrogenase is up regulated at the onset of nitrogen limitation in the growth medium of the oleaginous microorganism possibly as a means of trying to scavenge additional ammonium ions from intracellular materials**.**
Nitrogen limitation in the cultivation of an oleaginous microorganism induces a cascade of reactions leading to the formation of acetyl-CoA:
• At the onset of nitrogen exhaustion, oleaginous cells show an increased activity of AMP deaminase, which is fivefold greater than in cells before N limitation.
• The increased activity of AMP deaminase decreases the cellular content of AMP, including its content in the mitochondrion.
• The diminished content of AMP in the mitochondrion stops isocitrate dehydrogenase in oleaginous cells; this enzyme is strictly dependent on AMP for activity.
• As a result, isocitrate cannot be metabolized; it thus accumulates and is then readily equilibrated with citric acid (via aconites).
• Citrate therefore accumulates in the mitochondrion.
• An efficient citrate efflux system exists in the mitochondrial membrane for the export of citrate (in exchange for malate).
• Citrate enters the cytosol and is cleaved by ACL to give acetyl-CoA and oxaloacetate.
• The acetyl-CoA is used for fatty acid biosynthesis.
• The oxaloacetate is converted via malate dehydrogenase to malate, which is then used as the counter ion in the citrate efflux system.
Although, this metabolism of glucose to acetyl-CoA is able to account for the flux of the carbon substrate into fatty acid biosynthesis under nitrogen-limited conditions, it is not the complete story. Some microorganisms have been found in which ACL activity is present, though without the cells being able to accumulate lipid; however, the corollary is not true: no oil-accumulating microorganism has yet been reported that does not have ACL activity. Some other enzyme must be needed to ensure lipid accumulation.
Fatty acids are highly reduced materials and to achieve their synthesis as a ready supply of reductant as NADPH, is essential. The synthesis of 1 mol of a C 18 fatty acid requires 16 moles NADPH to be provided as 2 moles NADPH are needed to reduce each 3-keto-fattyacyl group arising after every condensation reaction of acetyl-CoA with malonyl-CoA as part of the standard fatty acid synthetase complex into the saturated fatty acyl chain, which then undergoes a further cycle of chain lengthening.

The major supplier of NADPH for fatty acid biosynthesis is now considered to be malic enzyme (reaction no. 3):

Malate +NADP→ pyruvate+ CO_2 +NADPH

Malic enzyme activity has been found in most oleaginous microorganisms to form an integrated metabolon complex that combines with ACL and fatty acid synthase (FAS) to ensure a direct channeling of acetyl-CoA into fatty acids, are finally esterified with glycerol into triacylglycerols and incorporated via the endoplasmic recticulum into fatty acid droplets (Figure 2).

Malic enzyme activity does not appear to be ubiquitous amongst oleaginous microorganisms and may be absent in some oleaginous yeasts, including *Lipomyces* sp.and some *Candida* sp. [unpublished work]. Here, it is probable that an alternative NADPH-generating enzyme, such as a cytosolic NADPH-dependent isocitrate dehydrogenase, dedicated to fatty acid biosynthesis much in the same way as malic enzyme is considered to be functionally associated with the fatty acid (Figure 2) metabolism (Ratledge and Wynn, 2002; Phillips and Huang, 1996). It is the opinion of the author and his colleagues that in oleaginous microorganisms there is no such thing as a general pool of NADPH, which can be produced by a number of enzymes; instead, for fatty acid biosynthesis we propose that there is an integration of the system for producing NADPH (e.g. malic enzyme) with the fatty acid synthesizing machinery. Only in this way can a concerted accumulation of triacylglycerols be achieved (Wynn *et al*., 1999).

Formation of polyunsaturated fatty acids

Fatty acid biosynthesis in almost all organisms culminates in the formation of either C 16 or C 18 saturated fatty acids. These fatty acids are then modified through a sequence of desaturases and elongases so that an extended range of unsaturated and PUFAs are produced.

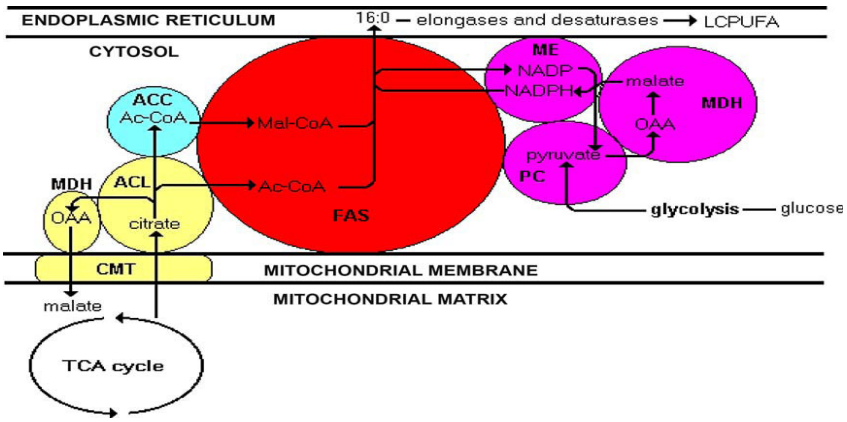


Figure 2
A diagram to show the organisation of a hypothesised lipogenic metabolon (Ratledge,2002).

Abundant production of fatty acids depends on the genetic makeup. The commercial interest has therefore concentrated on those fungi and micro algae that produce the high levels of desirable PUFAs coupled with the high contents of triacylglycerols. A repeated series of reactions incorporate acetyl moieties of acyl-CoA, resulting in chains length of 16 or 18 carbons. The enzymes involved in the synthesis are acetyl-CoA carboxylase and fatty acid synthase. Stearic acid, a C 18 saturated fatty acid, gets converted to oleic acid, a mono unsaturated fatty acid. The condensation is catalyzed by the enzyme Δ^9 desaturase. Δ^{12} desaturase converts oleic acid into LA, which further gets converted to ALA by the action of Δ^{15} desaturase. The conventional aerobic pathway, which operates in most (PUFA synthesizing) eukaryotic organisms, starts with Δ^6 desaturation of both 18:2 n-6 and 18:3 n-3,

resulting in the synthesis of γ-linolenic and octadecatrienoic acids, respectively. This, first desaturation step, is followed by Δ^6 specific C2 elongations to 20:3 and 20:4 and further Δ^5 desaturation to produce AA and EPA. The biosynthesis of DHA may follow two pathways. The first one is a linear pathway, involving C2 elongation of EPA to C22: 5, which are desaturated by Δ^4 specific desaturase to yield DHA. Another pathway, known as "Sprecher" pathway, is independent of Δ^4 desaturation. (Figure 3). It involves two consecutive C2 elongation cycles to yield 24:5, followed by Δ^6 desaturation and one cycle of C2 shortening via β oxidation in the peroxisome to yield DHA, Recent identification of a *Thraustochytrium* Δ^4 desaturase indicates that Δ4 desaturation indeed is involved in DHA synthesis. An alternative pathway for the biosynthesis of C20 PUFAs has been demonstrated in organisms that appear to lack Δ^6desaturase activity. The protist, *Tetrahymena pyriforms*, *Acanthamoeba* sp. and *Euglena gracilis,* synthesize PUFAs by this mechanism **(**Napier, 2003).

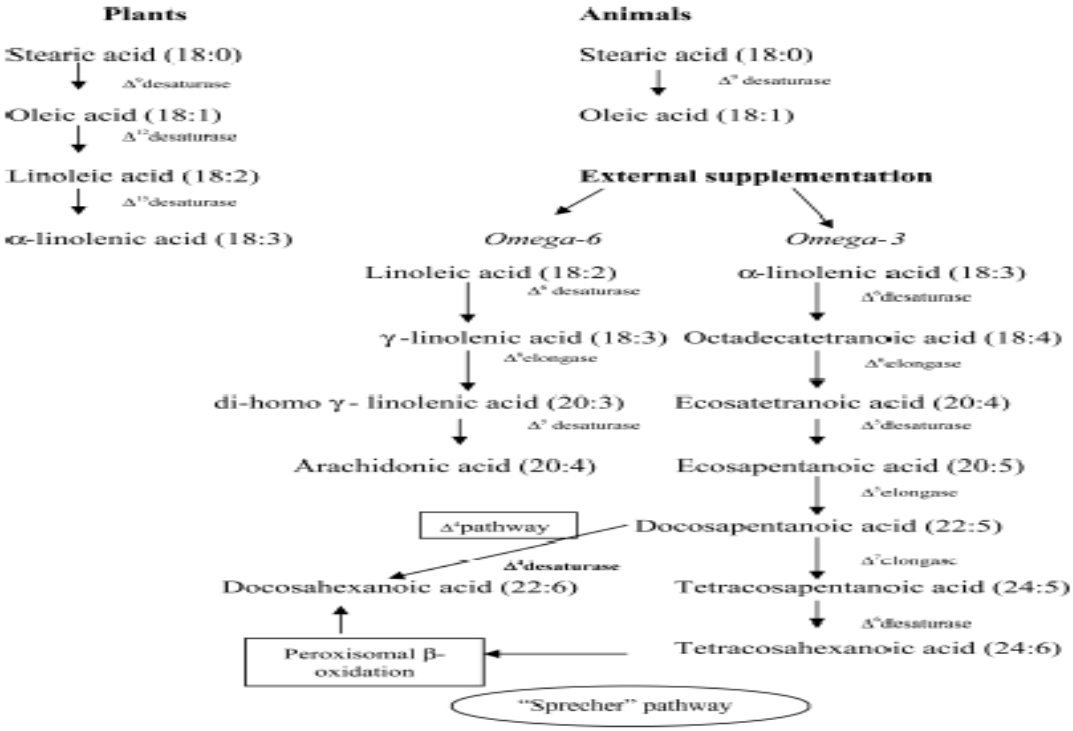


Figure 3
Schematic representation of fatty acid biosynthesis (Napier, 2003).

Regulation and control of microbial PUFA formation

Two basic processes have been developed for microbial production of PUFAs: submerged and solid state fermentations. 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. 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 (mutation, molecular engineering techniques, and the use of inhibitors, structuring microbial fatty acid composition by enzymatic treatment of pre-existing 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 (Certik, 1998). Several mutants with defective desaturases (A5, A6, A9, Al2 and o3 [Ar5, Al]) enhanced desaturase activities (A5) elongation defective mutants (Dittrich, 1998) or their combinations are employed for PUFA formation (Chattopadhyay, 1985; Anamnart, 1998; Ykema, 1990; Goodrich, 1995; Shimizu 1995). These mutants not only exhibit significantly improved production of naturally occurring PUFAs, but also from 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.

Genetic Engineering

Desaturases and elongases are the critical enzymes involved in the biogenesis of most of the PUFAs. During the last decade, several genes responsible for the biosynthesis of n-6 and n-3 PUFAs have been cloned from various organisms (algae, fungi, mosses, higher plants and mammals). Desaturases are iron-containing enzymes that introduce a double bond in a specific position in long chain fatty acids. There are two types of desaturases, omega and delta desaturases, both of which are membrane bound proteins. They create a double bond at a fixed position counted from the carbonyl end of the fatty acids, aerobically. This reaction requires molecular oxygen, NADPH, an electron transport system (ferredoxin-NADPH reductase and cytochrome b5) and a terminal desaturase. All the desaturases are acyl desaturases containing N terminal cytochrome b5 domain**,** which serves as an electron donor for their activity (Sperling,2003). These membranes bound desaturases and acyl coenzyme A (CoA) desaturases. The first group of enzymes is located in the membranes of thyalakoid, plant endoplasmic reticulum (ER) and plastid. The acyl lipid desaturases in cyanobacteria and plant plastid can desaturate stearic (18:0) and oleic (18:1) acyl groups in monogalactosyl diacylglycerol (cyanobacteria and plant plastid) and in phosphatidylglycerol (plant plastid), whereas plant ER desaturases mostly use fatty acids in phosphatidylcholine (Tocher, 1998). The other subgroups, acyl CoA desaturases are found to be present in ER membrane, which use fatty acyl-CoAs, as substrates. These are found to be present in animals, including insects and nematodes as well as in fungi. All the mammalian desaturases that have been identified are acyl–CoA desaturases. Several reports on cloning and characterization of desaturase genes are now available. In comparison, the work on the desaturase proteins is severely trailing, mainly because of problems in purifying them. Except Δ^9, other desaturase are membrane bound and pose technical problems in solubilising them. The structural characterization of the desaturase proteins is therefore poorly understood.

Enzymes that increase chain length are essential for biosynthesis of long chain polyunsaturated fatty acids. In n-3 and n-6 biosynthetic pathways, Δ^6 elongase converts arachidonic acid (18:4) to ecosatetraenoic acid (20:4) and GLA to ecosatrienoic acid (20:3) respectively. Genes for this condensing enzyme have been cloned from different organisms like moss *Physcomitrella patens* (Zank, 2002), *Isochrysis galbana*, a marine prymnesiophyte micro algae (Qi, 2002), the nematode *Caenorhabditis elegans*, (Beaudoin, 2000), liverwort *Marchantia polymorpha* (Kajikawa, 2004) and an zebra fish, *Danio rerio* (Agaba, 2004). It is interesting to note that substrate specificity of the Δ^6 elongase with respect to n-6 and n-3 pathway varies in different organisms. The elongase PSE1 from *P. patens* is more specific to the n-6 pathway rather than n-3, while genes coding for delta 6 elongase from *C.elegans* and *I.galbana* catalyze elongation in both pathways with similar magnitude. Elongase gene from zebra fish gives elongases that show specificity for all condensation reactions, viz.Δ^5,Δ^6,Δ^7. Fatty acid elongation can be divided into four different

reactions: condensation of malonyl –CoA, reduction to b-hydroxyacyl-CoA, dehydration to trans-2-enoyl-CoA, and reduction of the trans double bond resulting in the elongated acyl-CoA **(**Fehling, 1991; Dutta, 1996). The microsomal elongation system consists of four distinct enzymes: beta-ketoacyl-CoA synthase, a beta-ketoacyl-CoA reductase, a beta-hydroxyacyl-CoA dehydratase and an enoyl-CoA reductase catalyzing only a single step of the whole sequence.

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 A9 desaturase. Sterulic acid was efficiently used for the production of microbial oils having fatty acid composition similar to cocoa butter (Moreton, 1985). Various other cyclopropene fatty acid esters have been synthesized as a potential structure based inhibitors of A6, Ai* and Ai5 desaturases (Dulayymi, 1997). Three different types of inhibitors 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 non-oil fraction of sesame oil (Shimizu, 1991) and from Chinese medicine “Saishin” *(Asiasari radix) (*Shimizu, 1992*).* The sesamin analogues specifically and noncompetitively inhibit desaturase with *Ki* values in the range of 160- 710nM. None of the A6, A9, and Al2 desaturases found to be inhibited by low concentrations of these lignans. The second type involves alkyl gallate antioxidants which noncompetitively inhibit both A5 and A6 desaturases with *Ki* values of 26 PM and 170/IM**,** respectively (Kawashima, 1996). The inhibitory function requires special structural features such as the aromatic ring carries a hydroxyl group at the meta position and the carboxyl group esterified with an alcohol of appropriate carbon length. The third type composes of curcumin [1, 7-bis (Chydroxy-3-methoxyphenyl) - 1, 6-heptadiene-3, 5-Dione]**,** a major component of turmeric (Shimizu, 1992; Kawashima, 1996). It has a noncompetitive inhibitory effect on both AS *(Ki*= 36 PM) and A6 desaturase *(Ki= 28*) 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 A5 desaturase in fungi (Nakajima, 1993). Some analogues of fatty acids, trans-octadecenoic acids, were found to be competitive inhibitors of A9, A6, and A5 desaturases in rat liver microsomes. The herbicides of the substituted pyridazinone family showed inhibitory effects against A6 and w3 desaturases in microalgae (Cohen, 1992). On the other hand, Ca-channel blockers nicardipine and nifedipine inhibit rat microsomal desaturases noncompetitively (Kawasbima, 1996), while nicardipine specifically reduces A5 desaturase (Ki=62 FM), nifedipine efficiently blocks A6 desaturase (Ki=44 FM). Interestingly, when these two compounds were applied to fungal desaturases, no inhibition was observed. Conversely, verapamil, another Ca-channel blocker, reduces both microbial A5 and A6 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 the elucidation of desaturation complexes from various sources.

Enzymatic biotransformations

The application of lipase-catalyzed reactions in many industrial fields have led to the 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 co-products 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 (Gandhi, 1997). In particular, the enzymatic inter-esterifications 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 (Shimada, 1995; Huang, 1997; Wanasundara, 1998). 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 ω-3 PUFAs to the oils containing ω-6 PUFAs allows the preparation of oils with desirable ω-6/ω-3 PUFA ratios, leading to more beneficial human applications (Akob, 1996; Ju, 1998).

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, while 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 that causes many troubles is the failure to prevent lipolysis occurring during lipid recovery processing. Satisfactory treatment must also be used to minimize, auto oxidative 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 this point of view, the application of two-step extraction with ethanol and hexane leading to high yield of GLA-rich fungal oil is convenient and was also recommended for oil isolation from yeasts (Suzuki and Yokochi, US Patent 4 870 001, 1989; Certik, 1996; Davies, 1988). 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.*, (1990) investigated the supercritical fluid extraction using fungal model, but only under laboratory conditions. Recently, Certik and Horenitzky (1999) extended the process of supercritical CO_2 extraction of GLA-containing oil from the fungus *Cunninghamella echinulata* 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 and 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 such a way that as much as possible these compounds are retained in the oil. The 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 distill PUFAs or their esters, if this can be avoided

since they are liable to undergo double-bond migration, stereomutation, cyclization and dimerization under extreme thermal conditions, where distillation is unavoidable the artifacts 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 flavor 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 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 (Nkajima, 1993; Yokochi, 1989) and further purification by HPLC led to 99.5% GLA (Nakajima, 1993). Lipase-catalyzed selective esterification with n-butanol was efficiently applied to enrich GLA of fungal unesterified fatty acids from 10.4% to almost 70% (Mukherjee, 1991). In addition, high GLA recovery (90-95%), although originally applied to borage oil, was also obtained by treatment with acyl-specific fungal lipase, which yielded fatty acid with GLA content of 70 or 87% (Foglia, 1995; Huang, 1997). On the other hand, GLA ethyl esters of 98% purity were obtained from a mixture of fungal fatty acid esters using various types of Y-zeolites.

Commercial Development of GLA

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 IS-18% GLA (Ratledge**,** 1992C). Although, the oil after extraction and refining consisted of 98% TAG with twice the GLA content than evening primrose oil; marketing difficulties were the principal reasons 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 83g/L lipid (4.5% GLA in total fatty acids) representing 3.4g/L GLA (Nakabara, 1992). On the other hand, a new mutant of *Mortierella rammanniana,* grown in a highly viscous media fermentor equipped with a special mixing system, improved GLA production to 5.5 *g/*L (more than 30g/L lipid/l) with 18% GLA in oil (Hiruta, 1997). This oil is commercially used as a food additive or as a health food in the form of drinks, candy, jelly and the oil are also added to cosmetic preparations. Commercialized fungal GLA 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 (Certik, 1993). 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.,* (1993**),** who constructed a fungal model for the maximal GLA production and applied it to large-scale fermentations. To reduce the cost, the relationship between monocarboxylic acids (as waste materials from petrochemical processes) and GLA formation in fungi has been studied (Botha, 1997). Single cell oil rich in GLA (SCO) (15% w/w) was the first SCO to be produced commercially from *Mucor circinelloides* by JE Sturge, UK. This fungus is used in oriental food fermentations and it may be approved for human consumption. However, the prices of the latter plant oils remained stable or even decreased in such a way that SCO-GLA preparation became uncompetitive. The market is too small and specialized for a microbial product. There were several proponents of this approach in the mid-1970, But Dr John Williams founded Bio-Oils Research Ltd (Bio-Oils) and Dr David Horrobin founded Efamol Ltd (now Scotia Holdings plc). There are several forms of GLA supplements, which are sold under the trade name GLA 120, Tona lean and Star GLA. It is also available as

evening primrose oil, borage seed oil and blackcurrant seed oil. Doses tried for rheumatoid arthritis and other conditions range from about 360 mg to 2.8 g daily in divided doses and usually with meals. Doses of up to 2 g daily may be helpful in those with elevated triglycerides. The concentration of GLA varies in the different oil preparations and depending on the concentration desired doses of the number of capsules daily might be used.

Future Perspectives

Growing interest in PUFA applications in various fields coupled with their significane in health and dietary requirements has encourged "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, acyl transferases 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, which have 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 machinary 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 applications.

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