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Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae

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INTRODUCTION

Natural oils, fats and fatty acids, which can be derived from a variety of biological materials, are used for many different applications (Table 1). They are important in the food industry and also form the starting material for various industrial products like lubricants, plastics, coatings, detergents, soaps and cosmetics (1). This review will focus on the microbial production of long chain fatty acids (C_{18} and higher).

FATTY ACID BIOSYNTHESIS.

De Novo fatty acid synthesis: chain elongation and desaturation

In principle fatty acid synthesis involves the conversion of acetyl-CoA into the long chain fatty acid palmitate (C16:0). The first step is the formation of malonyl-CoA by carboxylation of acetyl-CoA, a reaction which is catalysed by an acetyl-CoA carboxylase (ACC). Subsequently 7 malonyl-CoA molecules are together with acetyl-CoA converted to palmitate (C16:0) by the fatty acid synthase (FAS) enzyme complex. The overall reaction involves different enzyme functions: acyl-carrier protein, acyl transacylase, malonyl transacylase, β-ketoacyl reductase, dehydratase and enoyl reductase. Basically there are two types of fatty acid synthases. Type I FAS is present in animals, birds, yeasts, fungi and some special bacteria. In this type all enzyme activities are located on one or two polypeptide chains (2,3,4). The second type, type II FAS, is present in plants, most bacteria and cyanobacteria. In type II FAS systems the enzyme activities are present on separate polypeptide chains (5). Palmitate (C16:0) formed via the FAS enzyme system can be elongated stepwise with 2 carbon atoms (from Malonyl-CoA) to C18, C20, C22 and C24 (3). For an overview of *de novo* synthesis of fatty acids in plants we refer to Slabas and Fawcett (6).

In the formation of unsaturated fatty acids another group of enzymes, the desaturases, are involved. The general desaturation process in yeasts is schematically depicted in fig.1 (adapted from 7). Most desaturases are membrane-bound multienzyme complexes. An exception are the fatty-acyl carrier protein (ACP) desaturases, found in plant chloroplasts, which are soluble enzymes (8). The first double bond introduced into an acyl chain is usually at the delta-9 position. In yeasts the reductase is a cytochrome b5 reductase, the electron donor is cytochrome b5 and the substrate is an acyl-CoA chain or an acyl-phospholipid. The

Table 1 Structure, origin and potential use of some natural fatty acids

Fatty acid	Structure	Source	Applications
Short chain	< C8	Palm kernel oil coconut oil	Plasticisers, alkyds, lubricants
Medium chain	C8 - C16	Cuphea, palm kernel oil, coconut oil	Surfactants, soaps, detergents, emulsifiers
Lauric acid	C12:0	Palm kernel oil	Soaps, detergents, margarine
Stearic acid	C18:0	Cocoa, shea	Confectionaries
Oleic acid	C18:1*c*9	Olive, rapeseed	Salad oil, cooking oil
Petroselenic acid	C18:1*c*6	*Coriandrum sativum*	Lauric acid + C6 acid
Ricinoleic acid	C18:1-OH	Castor oil	Lubricants, plastics
Vernolic acid	C18:1-O	Euphorbia oil	Cosmetics, pharmaceuticals,lubricants, Plasticisers, coatings, surfactants.
α-Linolenic acid	C18:3c3,6,9	Linseed	Paints, varnishes
γ-Linolenic acid (GLA)	C18:3*c*6,9,12	Evening primrose, moulds, fungi	Food-additive.
Arachidonic acid (ARA)	C20:4*c*5,8,11,14	Moulds, fungi	Food-additive.
Eicosapentaenoic acid (EPA)	C20:5*c*5,8,11,14,17	Moulds, fungi, algae, fish oil	Food-additive.
Erucic acid	C22:1*c*14	Rape oil	Anti-foam additive, perfume industry, plastics.
Docosahexaenoic acid (DHA)	C22:6*c*4,7,10, 13,16,19	Moulds, fungi, algae, fish oil	Food-additive, precursor eicosanoid hormones.

soluble desaturases from plant leaves and algae use stearoyl-ACP (the final product from their FAS) or fatty acids esterified to phospholipids as substrate, while ferrodoxin is the electron donor. Desaturases in plant cytosol use fatty acids esterified to phospholipids as substrate and cytochrome b5 as electron donor (9).

The best known desaturases are the Δ-9 desaturases, responsible for the conversion of C16:0 to C16:1*c*9 and of C18:0 to C18:1*c*9. A well studied delta-9 desaturase system in microorganisms is the Ole1 gene from *Saccharomyces cerevisiae*. This gene was cloned and expressed by Stukey *et al.* (10,11) and recently data on the regulation of the expression have been published (12,13,14). The work on the *S.cerevisiae* Ole-1 gene has facilitated the isolation and characterisation of the delta-9 desaturase genes from other yeasts like *Cryptococcus curvatus* (15,16) and *Pichia angusta* (17). Other desaturases are the Δ-12 desaturases involved in the subsequent desaturation of oleic

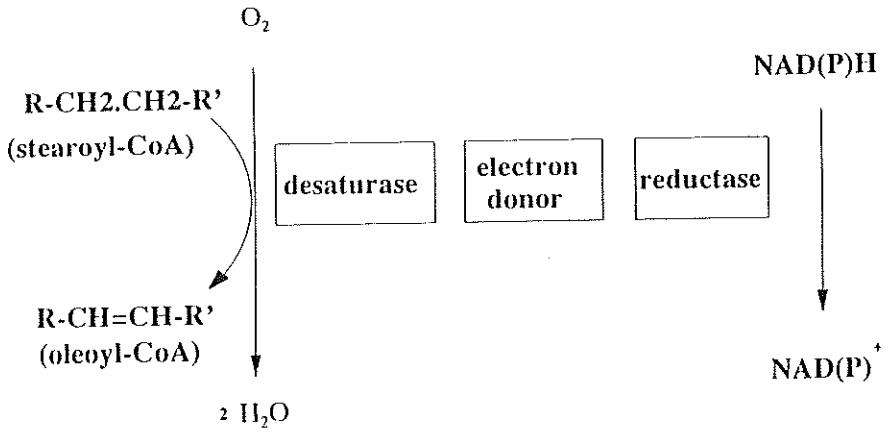

Figure 1. Schematical desaturation process of saturated fatty acids in yeast.

acid (C18:1*c*9) to linoleic acid (C18:2*c*9,12) and the Δ-6 and Δ-15 desaturases, responsible for the desaturation of linoleic acid to γ-linolenic acid (C18:3*c*6,9,12) and α-linolenic acid (C18:3*c*9,12,15), respectively. In addition to microbial desaturases, desaturase genes have been cloned also from plants (8).

Also several desaturase encoding genes from marine microorganisms have been studied. The genes for the Δ-12 (18,19), the Δ-6 (20), the omega 3 and the Δ- 9 acyl-lipid desaturases (21) from the cyanobacterium *Synechocystis sp.* and the Δ-6 desaturase gene from *Spirulina platensis* (22) have been cloned and characterized. Expression of the Δ-6 acyl lipid desaturase from *Synechocystis sp* in transgenic plants resulted in the production of γ-linolenic acid (23). Recently, the importance of (poly)unsaturation of membrane lipids in growth, respiration and photosynthesis was shown by targeted mutagenesis of the desaturases in *Synechocystis* (24).

After the synthesis of C16:0 or C18:0 fatty acids by the FAS system, the elongation and desaturation reactions can lead to fatty acids that are mostly present as phospholipids or as storage fatty acids in the form of triglycerides. An overview of fatty acid formation is depicted in figure 2 (modified from 9 and 25). In different groups of organisms presence, absence and differential regulation of enzym-activities leads to completely different fatty acid profiles.

NATURAL SOURCES OF FATTY ACIDS

Oil crops form the largest sources of fatty acids. Plants used for oil production synthesize FA up to C18:3 and rarely also to C20:1, C22:1 and C24:0 (25, 26, see also table 1). In order to produce specific FA in high amounts, transgenic plants have been developed. At present rapeseed varieties are available which contain oils with 40% lauric acid (C12:0; 26) and also plants with other designed oils are being developed (27, 28). The development of transgenic oilseed crops for commercial production of specific long chain fatty acids, however, which would involve the

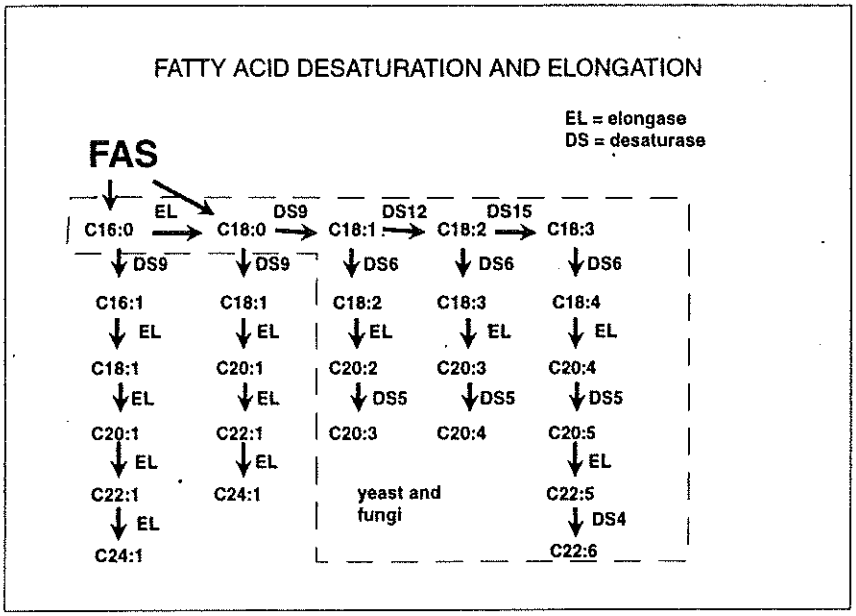

Figure 2. Metabolic pathways for the production of fatty acids

introduction and expression of several genes, is only expected in the next decades.

Apart from plants, oils can be obtained from microbial sources. Oil derived from microorganisms is referred to as Single Cell Oil (SCO). The industrial potential of SCO will be discussed in the paragraphs below.

Microorganisms are being considered oleaginous if they can accumulate lipid up to more than 20% of their cell dry weight (29). The group of oleaginous microorganisms includes mainly yeasts, fungi, moulds and algae. The oleaginous yeast *Crytococcus curvatus* (formerly described as *Candida curvata* or *Apiotrichum curvatum*) has been extensively studied in the past for the production of cacao butter equivalents (CBE; 30). *C. curvatus* is capable of utilising all kinds of substrates and displays excellent growth and lipid accumulation in fermenters (up to 60% CDW; 31, 32, 33). Other interesting oleaginous yeasts are for instance *Lipomyces starkeyi* and *Rhodotorula glutinis* (25, 32, 34). Fungi or moulds that have attracted industrial attention include members of the order *Mucorales* (25) and members of the genus *Mortierella* (35, 36), which all produce γ-linolenic acid (GLA). Arachidonic acid (ARA) and other PUFAs are found in algae, mosses (32, 37) and also in *Mortierella* species (38, 39, 40). An overview of most organisms able to produce SCO is given by Kyle and Ratledge (41).

OPPORTUNITIES FOR SINGLE CELL OILS PRODUCTION PROCESSES

As the prices for most bulk plant oils are relatively low, and animal fats are even cheaper, there is no

prospect that a microbial oil similar to these could ever be produced economically.

Attempts to produce CBE equivalents by *C.curvatus* (42) and GLA by *Mucor circinelloides* or *Mortierella isabella* (36, 43) were technically feasible but have not been an economical success (25).

However, there are still significant opportunities for Single Cell Oil production since:

- From clinical research it becomes more and more evident that mammals (especially infants) have a dietary need for long chain polyunsaturated fatty acids (LCPUFAs , $>C_{20}$, 2 or more double bonds). These fatty acids are not generally present in plants but are produced by moulds and micro algae.
- Genetic modification of plants producing special fatty acids like the LCPUFAs will be a very difficult and time consuming task since it requires the introduction of several genes.

Genetically modified oleaginous yeasts may fill the gap between microorganisms like moulds and algae which produce very interesting long chain FA but are relatively difficult to cultivate in large amounts, and plants. In addition, the expertise and tools developed for the genetic modification of microorganisms may be used for the development of transgenic plants at a longer term. At present, for instance, desaturation genes from *Synechocystis* already have been expressed in plants. This led to the production of gamma-linolenic acid (23) and to cold resistance (44). Introduction of a yeast Δ-9 desaturase gene in tobacco led to a change in fatty acid profile (45,46) and expression of the same gene in tomato changed the composition of flavour compounds (47). Expression of a *S. cerevisiae* acyltransferase gene in Brassica results in modification of the seed oil content and the acyl composition (48). Alternatively yeast can serve as a model for expression of plant genes. For instance, the FAD2 gene from Arabidopsis was functionally expressed in *S.cerevisiae* (49).

LONG CHAIN POLYUNSATURATED FATTY ACIDS

LCPUFAs are classified according to the location of the first double bond. Thus, omega-3 (n-3 or ω3) PUFAs have the first double bond three carbon atoms from the methyl end of the molecule, and omega-6 (n-6 or ω6) PUFAs have the first double bond six carbon atoms from the methyl end. The parent ω3 fatty acid is α-linolenic acid (LNA; C18:3ω3) and the parent ω6 fatty acid is linoleic acid (LA; C18:2 ω6). In humans, LNA is metabolized to eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:6 ω3), although at a lower rate, and LA is metabolized to arachidonic acid (ARA, C20:4 ω6). Recently the interest in ARA, EPA and DHA has increased enormously since it has become apparent that they have distinct physiological effects in the body (50).

ω-6 PUFAs are essential structural components of phospholipids in the cell membrane. There, they affect membrane characteristics and functions like fluidity, electrolyte transport and hormonal and immunological activities. In particular dihomo gamma linolenic acid (DHGLA) and ARA are also precursors of thromboxanes, prostoglandins and leukotrienes (51), which are biologically active in very small quantities. Applied in cosmetics or taken orally, ω-6 PUFAs (mainly GLA) have often helped to improve symptoms of skin disorder.

ω-3 PUFAs (e.g. α-linolenic acid, eicosapentaenoic acid [EPA], docosahexaenoic acid [DHA]), too, are essential components of cell-membrane phospholipids. At present, there is accumulating evidence that the ω-3 PUFAs may

reduce or inhibit risk factors involved in cardiovascular disease (52, 53), as well as inflammatory and immune disorder (54).

Although optimal intake of PUFA has not yet been established, the British Nutrition Foundation recommends a PUFA intake of 7.5% of total calories and a ω-6 to ω-3 PUFA ratio between 5:1 and 3:1 (55).

With respect to infant nutrition it becomes evident that long chain ω-3 PUFA, and especially DHA, are essential for fetal growth and infant development (56). Breast milk serves as a good source of long chain fatty acids. Infant formulas, however, in general are devoid of these fatty acids (57). As infants are not able to synthesize DHA at a rate fast enough to keep up with the demand from the rapid growing brain (58), it has been recommended that all infant formulas, as well as parental preparations include ω-3 PUFA (59).

SOURCES OF LCPUFAS

Traditional sources

Linoleic acid can be obtained from seeds from corn, cotton, safflower and soya (LA) whereas the main sources of GLA (18:3 ω6) are borage (starflower), evening primrose and blackcurrant (60). Green vegetables like cabbage, spinach, broccoli, lettuce, etc provide most of the alpha-linolenic acid in our diet. Longer fatty acids (including DHA and relatively large amounts of EPA), are present in fatty fish like herring, mackerel, sardine and salmon (60). The application of LCPUFAs from fish oils in foods or for inclusion in infant formula, however, has some draw backs in terms of overall output and product quality. They are often unsuitable because of contamination of fish by environmental pollution and problems associated with the typical fishy smell and unpleasant taste. In addition, fish oils generally contain EPA, an undesirable component in infant formulas because of its depression of arachidonic levels in infants. This has been correlated with reduced rates of infant weight gain (61).

Due to the growing evidence of the importance of PUFAs in human nutrition as well as their benefit in pharmaceutical application it is expected that in the near future the production of polyunsaturated fatty acids above C20 from current sources is inadequate for supplying the expanding PUFA market (62) In order to meet these expected demands, other, microbial sources are now actively being sought for commercial, large scale production of PUFAs.

Non traditional PUFA sources

Two approaches can be followed to develop new microbial PUFA production processes

1. Screening of microorganisms for the production of PUFAs and optimization of the production processes.
2. Metabolic engineering of oil producing microorganisms in order to obtain the desired product, followed by process optimization.

In the following paragraphs we will focus on two microbial systems which are examples of the two approaches. Firstly,we will discuss the possibilities of production of LCPUFAs by algae. The emphasis of this example will be on DHA production. Secondly, we will present recent advances made with the oleaginous yeast *Cryptococcus curvatus*, which seems a very promising model and production system for the study of fatty acid modification and for the production of special fatty acids.

Natural PUFA producing microorganisms

Several marine microorganisms including phytoplankton, seaweeds and fungi are capable to

produce DHA and/or EPA (63, 64, 65, 66). Of the limited number of microalgal species so far analysed for their lipid composition, an appreciable proportion displayed fatty acid profiles that differed from the typical composition of higher plants in containing significant levels of LCPUFAs. (64). However, there are several difficulties, with respect to optimal growth conditions, large scale production and extraction and formulation processes to overcome, in order to produce LC-PUFA on a large scale from many of these organisms. In general the scheme as depicted in fig 3 can be used to develop the process (67). Within this approach also the effect of the different cultivation and processing steps on product quality and product quantity should be included as these parameters largely determine the final price of the product.

The most promising microorganisms for the production of DHA are microalgae and marine fungi (63, 68, 69). At present, DHA containing oils are economically produced by using the heterotrophic alga *Crypthecodinium cohnii* (69). An axenic culture of *C. cohnii* is cultivated in large scale fermenters and environmental parameters like temperature, pH, air flow, pressure, agitation and dissolved oxygen are well controlled. The medium typically used for growth of *C. Cohnii* consists of NaCl, $CaCl_2$, $MgSO_4$, dextrose and yeast extract or hydrolysed vegetable protein (69). Under controlled laboratory conditions cell densities up to 40 g/l may be attainable in about 5 days (68). The oil content of the cell is between 15 and 30% with between 20 to 35% DHA and very low amounts of other polyunsaturated fatty acids (68, 70). The absence

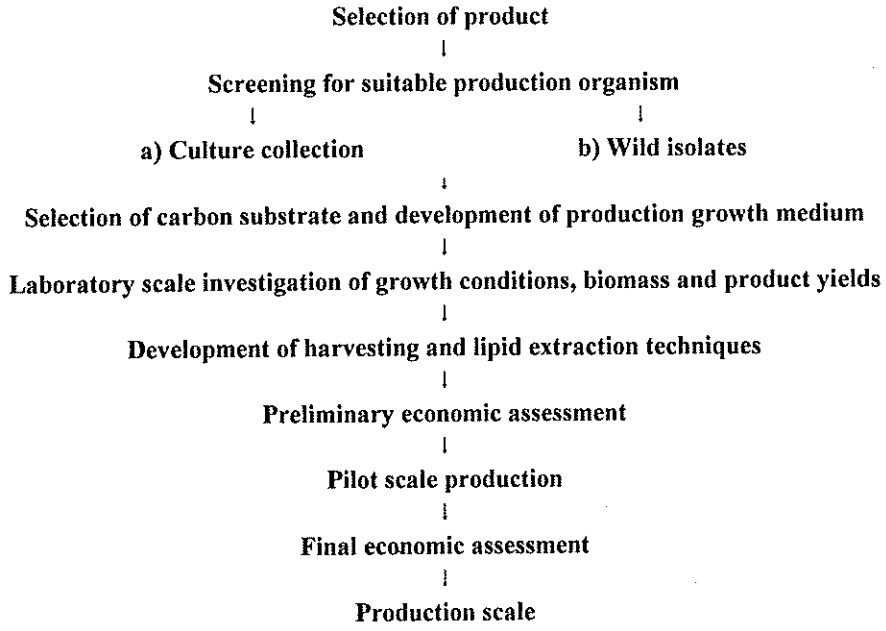

Figure 3. Strategy for the development of new microbial products

or low amount of other polyunsaturated fatty acids in oils obtained from *C. Cohnii* facilitates the down stream processing.

SINGLE CELL OIL PRODUCTION WITH THE OLEAGINOUS YEAST *CRYPTOCOCCUS CURVATUS*

Isolation and classification of *Cryptococcus curvatus*

The oleaginous yeast *C.curvatus* was first isolated by Moon *et al.* (71) and identified as *Candida curvata D.* During the past 18 years the classification of this yeast has been changed a few times. The American Type Culture Collection (ATCC; Rockville, Md., USA) classified it as *Apiotrichum curvatum* (ATCC 20509). Recently the Centraal Bureau Schimmelcultures (CBS) (Delft, The Netherlands) stated this yeast to be a *Cryptococcus curvatus*. In this review the name *Cryptococcus curvatus*, according to the latest CBS classification, will be used.

Optimal growth temperature and pH for *C. curvatus* are 28°C and pH 5.4, respectively (71). Evans and Ratledge (72) observed growth and lipid accumulation on a variety of sugars like glucose, sucrose, lactose and xylose. Growth was also demonstrated on fatty acids or oils as a substrate (73) and on various waste materials like whey permeate (33), beet molasses (74), banana juice (75) and prickly pear juice (76).

High-cell density cultivation of *C. curvatus*

For the cultivation of oleaginous yeasts a fed-

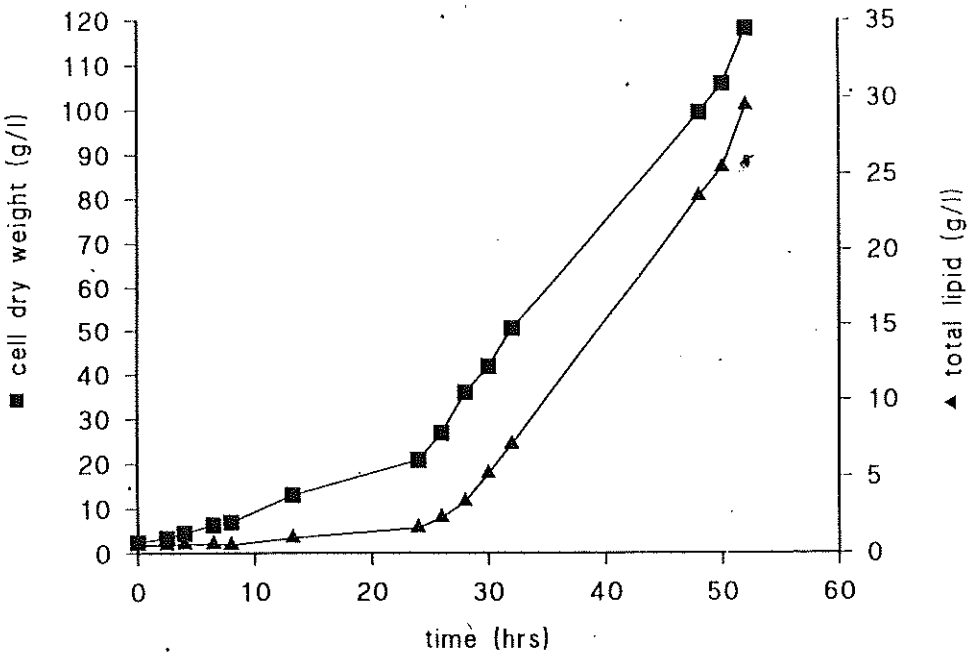

Figure 4. Biomass and lipid production of *C. Curvatus* grown on glycerol in a fed-batch system.

batch cultivation mode has proven to be very useful. Very high cell densities have been reached with *Lipomyces starkeyi* (153 g/l; 77) or *Rhodotorula glutinis* (185 g/l; 78). Ykema (33) and Granger *et al.* (79) showed the importance of a high C/N ratio for lipid accumulation. The best results should be expected with C/N greater than 20. In the fed-batch fermentation we performed, a two-stage approach was followed. In the first stage cells were grown without nitrogen limitation. Substrate was fed in the form of a 87% glycerol solution, using a sudden increase of the oxygen tension as a signal for feeding (80). In the second stage a nitrogen limitation was applied by controlling the pH with 5 N NaOH instead of ammonia. In figure 4 the amount of biomass and total lipid during 52 h of fermentation in a 2-l fermenter is shown. The overall yield on glycerol was 0.40 g biomass/g substrate, the lipid yield was 0.11 g lipid produced/g substrate and the overall lipid production rate of the fermentation was 0.59 g lipid/l/h. After 32 h of cultivation NaOH instead of ammonia was used to control the pH. During the nitrogen limited growth phase 23 g lipid/l was accumulated in 20 h, corresponding to a lipid production rate of 1.15 lipid/l/h.

Yeast cells, collected during the fermentation, were analysed for oil content and fatty acid composition. When the yeast cells were growing linearly, no oil accumulation occurred. In this stage all fatty acids (±10% of cell dry weight) are derived from membrane lipids. In Table 2 the amounts of fatty acid in the storage lipids are shown (recalcultated from ref. 81). During lipid accumulation the amount of C18:2 decreases and the amounts of C18:1, C18:0 and C16:0 increase, resulting in a significant change in the C18:2 / C18:1 ratio. These data demonstrate that the accumulated storage lipids contain mainly C16:0, C18:0 and C18:1 fatty acids .

Table 2 Amount of fatty acids of *C. curvatus* cells during fermentation

	Amount of FA in storage lipids (g/l)				
Cultivation time (h)	C16:0	C16:1	C18:0	C18:1	C18:2
8	0	0	0	0	0
32	0.3	0	0.8	1.4	0.8
52	3.9	0	4.2	10.3	0.5

METABOLIC ENGINEERING OF FATTY ACID BIOSYNTHESIS IN *C.CURVATUS.*

Due to economical aspects, single cell oil processes will only be feasible when high-priced lipids and fatty acids with unique chemical, physical or nutritional properties, e.g. LCPUFAs, can be produced. As *C.curvatus* is a fast growing microorganism that is able to accumulate large amounts of oil, this yeast has potential for the production of these high priced products.

The production of PUFAs, however, requires the introduction and expression of one or more genes in *C.curvatus*.

Until recently no expression and transformation system was available for *C. curvatus* and therefore molecular biological techniques could not be applied. At present, however, we have developed a transformation system based on selection on phleomycine (82) thereby opening the way to genetically modify the fatty acid composition in this oleaginous yeast.

To allow genetic modification of the fatty acid biosynthesis routes in *C.curvatus*, the Δ-9 fatty acid desaturase gene was cloned and characterized from two strains. The 1668 bp gene from strain ATCC 20509, encoding a protein of 556 amino acids with a calculated molecular mass of 62 kDa, showed strong homology to the gene from

C.curvatus strain CBS570 and to previously cloned Δ-9 fatty acid desaturase genes from *Saccharomyces cerevisiae* (10,11) and rat (83). Homology includes three histidine boxes characteristic for membrane bound desaturases and a cytochrome b_5 domain responsible for electron transport. The Δ-9 desaturase gene has a high G/C content of 61% and displays a codon usage different from *S. cerevisiae*, but similar to the basidiomycete *Schizophyllum commune*.

Expression of the Δ-9 desaturase gene of *C.curvatus* ATCC 20509, was studied in the presence of different fatty acids in the growth medium. Repression of desaturase mRNA signals was found if cis fatty acids with a double bond at the Δ-9 position were present. Therefore it was concluded that the Δ-9 fatty acid desaturase is regulated at transcription level (7,16).

Table 3. Input variables for cost estimations of the three production processes of SCO containing 40 % DHA. Abbreviations: CDW; cell dry weight, Tferm; total fermentation time, Lip; percentage lipid of the cell dry weight; DHA/Lip, percentage DHA of the lipids.

	CDW (g/l)	Tferm (h)	Lip (%)	DHA/Lip (%)
T. aureum	5	144	20	50
C. cohnii	40	120	25	40
C. curvatus	118	50	50	40

ECONOMIC CONSIDERATIONS FOR MICROBIAL PUFA PRODUCTION

For the development of good single cell oil (SCO) production processes economic feasibility calculations have to be performed. They do not only play an important role in the decision making but are also very helpful in determining where research attention should focus on.

In this paragraph the costs of three different processes for the production of SCO containing 40% DHA are estimated. The processes differ in the micro organism used. The fungus *Thraustochytrium aureum* (63) and the alga *Crypthecodinium cohnii* (68) naturally produce DHA. In the third process it is assumed that the oleaginous yeast *Cryptococcus curvatus* (15, 16, 81) is modified in such a way that it is able to produce DHA. In table 3, the input variables are shown for the rough cost estimations of these three production processes.

The calculations were performed according to a modified method of Lee *et al.* (84). In all three cases a similar production plant is assumed. The costs in US$ per gram of the SCO (CG), the annual SCO production in tonnes (YSCO) and the relative contributions in percentages of: the chemicals (RCC), the direct fixed capital dependent costs (DFC), the labour dependent items (LDI) and other costs (Other) are shown in table 4.

Table 4. The costs in US$ per gram of the SCO (CG), the yearly SCO production in tonnes (YSCO) and the relative contributions in percentages of the chemicals (RCC), the direct fixed capital dependent costs (DFC), the labour dependent items (LDI) and other costs (Other).

	CG US$/kg	YSCO ton/year	RCC (%)	DFC (%)	LDI (%)	Other (%)
T. aureum	960	5.70	5	46	18	31
C. cohnii	140	53.3	30	40	13	17
C. curvatus	23	612	62	18	7	13

These estimations show that the costs per kg of the DHA containing single cell oils are US$ 960 when produced with *T. aureum* and US$ 140 when *C. cohnii* is used. The yearly SCO productions using *T. aureum* and *C. cohnii* are 5.7 tonnes and 53.3 tonnes, respectively. The highest SCO productivity can be achieved with *C. curvatus*, 612 ton/year. In this case the costs per kg are the lowest, US$ 23. These data show that the product costs largely depend on the productivity of the microorganisms.

Not only the costs per gram product change at increasing productivities but also the relative contributions of the different cost factors. In case of production with *T. aureum* only 5% of the costs are represented by the chemical costs while for the more productive *C. curvatus* process the costs are mainly determined by the costs of the chemicals (62 %).
Therefore, during the development of a process in the low productivity regions, research should be less concerned about the substrate costs and more occupied in trying to increase the productivity. At high productivities efficient substrate usage becomes much more essential.

FUTURE OUTLOOK

In order to reach fatty acid profiles which will allow economical production, genetic methods offer attractive opportunities. This in turn requires a comprehensive understanding of the biosynthetic pathways of PUFAs in terms of intermediates and enzymes. For *C.curvatus* the possibility of genetic modification opens the door to perform research on these pathways by means of introducing genes in the wild-type yeast and to do gene disruption analysis and studie the effects on oil accumulation. The production of tailor-made high priced fatty acid may be the result of this research and on the longer term this knowledge can be implemented in genetic modification of oil crops for the production of bulk quantities of desired fatty acids.

REFERENCES

1. Röbbelen, G. 1989. In: De Bont JAM (ed), Biotechnology and fatty acids: new perspectives for agricultural production?, Pudoc, Wageningen, The Netherlands.
2. Schweizer, M., Lebert, C., Holtke, J., Roberts, L.M. & Schweizer, M. 1984. Mol. Gen.194, 457.
3. Schweizer, E. 1989. In: Ratledge, C. and Wilkinson, S.G. (eds), Microbial lipids Vol.2, p 3, Academic press London, UK..
4. Schweizer, E. 1996. Naturwissenschaften, 83, 347.
5. Topfer, R. & Martini, N. 1994. J. Plant Physiol. 143, 416.
6. Slabas, A.R. & Fawcett, T. 1992. Plant Molecular Biology 19, 169.
7. Meesters, P.A.E.P. 1997. Phd. Thesis V.U. Amsterdam. The Netherlands.
8. Sommerville, C. & Browse, J. 1996. Trends in Cell Biology 6, 148.
9. Harwood, J.L. 1994. In: Gunstone, F.D, Harwood, J.L. & Padley, F.B.(Eds) The lipid Handbook, Chapter 11, Chapman & Hall, U.K.
10. Stukey, J.E., McDonough, V.M. & Martin, C.E. 1989. J. Biol. Chem. 264, 16537.
11. Stukey, J.E., McDonough, V.M. & Martin, C.E. 1990. J. Biol. Chem. 265, 20144.
12. McDonough, V.M.,Stukey, J.E. & Martin, C.E. 1992. J. Biol. Chem. 267, 5931.
13. Choi, J.Y., Stukey, J., Hwang, S.Y. & Martin, C.E. 1996. J. Biol. Chem. 271, 3581.
14. Gonzales, C.I. & Martin C.E. 1996. J. Biol. Chem. 42, 25801.
15. Meesters P.A.E.P. & Eggink G. 1996. Yeast 12, 723.
16. Meesters, P.A.E.P., Springer, J. & Eggink, G. 1997. Appl Microbiol Biotechnol 47, 663.
17. Anamnart, S., Tomita, T., Fukui, F.,

Fujimori, K., Harashima, S., Yamada, Y. & Oshima Y. 1997. Gene 184, 299.
18. Wada, H., Gombos, Z. & Murata, N. 1990. Nature, 347, 200
19. Sakamoto, T., Wada, H., Nishida, I., Ohmori, M. & Murata, N. 1994. Plant Mol. Biol. 24, 643.
20. Reddy, A.S., Nuccio, M.L. Gross, L.M., & Thomas, T.L. 1993. Plant Mol. Biol. 22, 293
21. Sakamoto, T. & Bryant, D.A. 1997. Mol. Biol 23,1281
22. Murata, N., Deshnium, P. & Tasaka, Y. 1996. In: Huang, Y. and Milles D.E. (Eds), Gamma-linolenic acid, metabolism and its role in nutrition and medicine, p22, OACS Press, Champain, Illinois,
23. Reddy, A.S. & Thomas, T.L. 1996. Nature Biotechnol. 14, 639.
24. Tasaka, Y., Gombos, Z., Nishiyama, Y., Mohanty, P., Ohba, T., Ohki, K. & Murata, N. 1996. EMBO Journal 15, 6425.
25. Ratledge, C. 1993. Tibtech 11, 278.
26. Murphy, D.J. 1996. Tibtech 14, 206.
27. Kinney, A.J. 1996. Nature Biotechnol. 14, 946.
28. Budziszewski, G.J., Croft, K.P.C. & Hildebrand, D.F. 1996. Lipids 6, 557.
29. Ratledge, C. 1991. Acta Biotechnol 11, 429.
30. Ykema, A., Verbree E.C., Verwoert, I.I.G.S., Linden van der K.H., Nijkamp, J.J. & Smit, H. 1990. Appl. Micrbiol. Biotechnol. 33, 176.
31. Ratledge, C. 1989. Microbial lipids: prospects for biotechnology. In: De Bont JAM (ed), Biotechnology and fatty acids: new perspectives for agricultural production?, p 52, PUDOC, Wageningen, The Netherlands.
32. Rattray J.B.M. 1984. JAOCS 61, 1701.
33. Ykema, A. 1989. PhD thesis Free University Amsterdam, The Netherlands
34. Rattray, J.B.M. 1988. Yeasts. In : Ratledge, C. & Wilkinson, S.G. (eds), Microbial Lipids Vol.1, p 555, Academic Press, London (UK).
35. Buráňová, L., Řezanka, T. & Jandera, A. 1990. Folia Microbiol. 35, 578.
36. Preez du, J.C., Immelman, M, Kock, J.F.L & Kilian, S.G. 1995. Biotechn. letters 17, 933.
37. Radwan, S.S. 1991. Appl. Microb. Biotechnol. 35, 421.
38. Eroshin, V.K, Dedyukhina, E.G, Chistyakova, T.I, Zhelifonova, V.P. & Botast, R.J. 1996. Microbiology 65, 31.
39. Singh, A. & Ward, O.P. 1997. Appl. Microbiol. Biotechnol. 48, 1.
40. Kawashima, H., Nishihara, M., Hirano, Y., Kamada, N., Akimoto, K., Konishi, K. & Shimizu, S. 1997. Appl. Environ. Microbiol. 63, 1820.
41. Kyle, D.J. and Ratledge, C. 1992. Industrial Applications of Singe Cell Oils, Am. Oils Chem. Soc. Champaign, Illinois.
42. Davies, R.J. 1992. In: Kyle D.J. & Ratledge C. (eds), Industrial applications of single cell oils. pp 196, American oil chemists' society, Champaign, Illinois.
43. Nakahara, T., Yokocki, T., Kamisaka, Y., & Suzuki. 1992. In: Kyle D.J. & Ratledge C. (eds), Industrial applications of single cell oils. pp 61, American oil chemists' society, Champaign, Illinois.
44. Ishizaki-Nishizawa, O., Fujii, T., Azuma, M., Sekiguchi, K., Murata, N., Ohani, T. & Toguri, T.1996. Nature Biotechn. 14, 1003.
45. Grayburn, W.S., Collins G.B. & Hildebrand D.F. 1992. Bio/Technology 10, 675.
46. Polashock, J.J., Chin C.K. & Martin C.E. 1992. Plant Physiol. 100, 894.
47. Wang, C., Chin C.K., Ho C.T., Hwang C.F., Polashock, J.J. & Martin C.E. 1996. J. Agric. Food Chem. 44, 3399.
48. Zou, J., Katavic, V., Giblin, M., Barton, D.L., MacKenzie, S.L., Keller, W.A., Hu, X. & Taylor, D.C. 1997. The Plant Cell, 9, 909.
49. Kajiwara, S., Shirai, A., Fujii, T., Toguri, T., Nakamura, K. & Ohtaguchi K. 1996. Appl. Environ. Microbiol. 62, 4309.
50. Gibson, R.A., Makrides, M. & Sinclair, A.J.

1996. Lipids 31:51-119. (Proceedings of A.O.C.S. conference of 1994 containing 10 position papers regarding the potential use and benefits of including DHA in infant formula).
51. Specher, H. 1981. Prog. Lipid Res. 20, 13.
52. Kang, J.X & Leaf, A. 1996. Lipids 31, SA41.
53. Kromann, N. & A. Green. 1980. Acta Med. Scand. 208, 401.
54. Kremer, J.M. 1996. Lipids 31, S243.
55. British Nutrition Foundation: 1992. In: Unsaturated Fatty Acids, Nutritional and Physiological Significance. The Report of the Britisch Nutrition Foundation's Task Force, p 152, Chapman & Hall, London, U.K.
56. Innis, S.M. 1991. Prog. Lipid Res. 30, 39.
57. Huisman, M., van Beusekom, C.M., Lanting, C.I., Nijeboer, H.J., Muskiet, F.A.J. & Boersma, E.R. 1996. Eur. J. Clin. Nutr. 50, 225.
58. Crawford, P. 1987. In: Lands, W.E.M. (ed), Proc. Amer. Oil Chem. Soc. Short Course in Polyunsaturated Fatty Acids and Eicosanoids, pp 270-295, Amer. Oil Chem. Soc.
59. FAO/WHO Expert committee. 1994. Food and Nutrition paper No 57. FAO, Rome, Italy.
60. Gunstone, F.D. 1996. Fatty acid and lipid chemistry, Blackie Academic, London
61. Carleson, S.E., Cooke, R.J., Werkman, S.H., Peeples, J.M., Tolley, E. & Wilson, W.M.. 1990. Inform 1:306.
62. Gill, I. & Valivety, R. 1997. Tibtech 15, 410...
63. Bajpai, P., Bapai, P.K. & Ward, O.P. 1991. Appl. Microbial Biotechnol. 35, 706.
64. Cohen, Z., Norman, H.A. & Heimer, Y.M. 1995. In: Simopoulos, A.P. (ed), World Rev. Nutr Diet. 77, 1, Basel, Karger.
65. Gunstone, F.D, Harwood, J.L. & Padley, F.B. 1994. The lipid Handbook. Chapman&Hall, U.K.
66. Kendrick, A. & Ratledge, C. 1992. Lipids 27, 15.
67. Moreton, R.S. 1988. In: T.H. Applewhite, T.H. (ed). Proceedings World conference on biotechnology for the fats and oils industry. Am. Oil Chem. Soc.
68. Kyle, D.J., Sicotte, V.J., Singer, J.J. & Reeb, S. In: Kyle, D.J. & Ratledge, C. (eds.) Industrial Applications of Singe Cell Oils, p287, Am. Oils Chem. Soc. Champaign, Illinois.
69. Kyle, D.J. 1996. Lipid Techn. September 107.
70. Henderson, R.J., Lefteley, J.W. & Sargent, J.R. 1988. Phytochem. 27, 1679.
71. Moon, N.J., Hammond, E.G. & Glatz, B.A. 1978. J. Diary Sci. 61, 1537.
72. Evans, C.T. & Ratledge, C. 1983. Lipids 18, 623.
73. Lee, I., Hammond, E.G. & Glatz, B.A. 1992. In Kyle, D.J. and Ratledge, C. (Eds), Industrial applications of single cell oils. pp. 139, Am. Oils Chem. Soc. Champaign, Illinois
74. Bednarski, W, Leman J. & Tomasik, J. 1986. Agricultural Wastes 18, 19.
75. Vega, E.Z., Glatz B.A. & Hammond E.G. 1988. Appl. Eviron. Microbiol.54, 748.
76. Hassan, M., Blanc P.J., Granger L.M., Pareilluex A. & Goma G. 1996. Process Biochemistry 31, 355.
77. Yamauchi H., Mori, H., Kobayashi ,T. & Shimizu, S. 1983. J. Ferm.Technol. 61, 275.
78. Pan J.G., Kwak, M.Y. & Rhee J.S. 1986. Biotechnol. Letters 8, 715.
79. Granger L-M, Perlot, P., Goma, G. & Pareilleux, A. 1993. Appl Microbiol. Biotechnol. 38, 784
80. Yano, T., Kurokawa, M. & Nishizawa, Y. 1991. J. Ferm. Bioeng, 71, 345.
81. Meesters, P.A.E.P. & Huijberts, G.N.M. 1996. Appl. Microbiol. Biotechnol. 45, 575.
82. Springer, J. and Eggink, G. 1997, Manuscript in preparation.
83. Thiede M.A., Ozols J. & Strittmatter, P. 1986. J. Biol. Chem. 261, 13230.

84. Lee, S.Y., Choi, J. & Chang, H.N. 1996. In: Eggink, G. Steinbuchel, Poirier, Y. & Witholt, B. (eds), International symposium on bacterial polyhydroxyalkanoates, p127, Davos, Switserland.