Docosahexaenoic acid production by the marine alga *Crypthecodinium cohnii*

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Proefschrift

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Chapter 1

General introduction

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Structural diversity and physiological functions of fatty acids and lipids

Molecular structure of fatty acids and lipids

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 (Table 1) are unbranched and consist of an even number of carbon atoms (10-24). They may have various degrees of unsaturation (0-6 double bonds).

Unbranched fatty acids can be described in short by y:x, where y represents the number of carbon atoms and x the number of double bonds. Carbon atoms are counted from the carboxyl terminus. The positions of the double bonds are represented by Δ and a number. For example, Δ9 18:1 denotes a fatty acid with 18 carbon atoms and with one double bound at position 9 as counted from the carboxyl terminus. The configuration of most double bonds in naturally occurring unsaturated fatty acids is *cis*.

Table 1 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	Δ9-Hexadecenoic acid	Δ9 16:1
Oleic acid	Δ9-Octadecenoic acid	Δ9 18:1
ω-6 Polyunsaturated fatty acids		
Linoleic acid (LA)	Δ9, Δ12-Octadecadienoic acid	ω-6 18:2
γ-Linolenic acid (GLA)	Δ6, Δ9, Δ12-Octadecatrienoic acid	ω-6 18:3
Arachidonic acid (ARA)	Δ5, Δ8, Δ11, Δ14-Eicosatetraenoic acid	ω-6 20:4
ω-3 Polyunsaturated fatty acids		
α-Linolenic acid (LNA)	Δ9, Δ12, Δ15-Octadecatrienoic acid	ω-3 18:3
Eicosapentaenoic acid (EPA)	Δ5, Δ8, Δ11, Δ14, Δ17-Eicosapentaenoic acid	ω-3 20:5
Docosahexaenoic acid (DHA)	Δ4, Δ7, Δ10, Δ13, Δ16, Δ19-Docosahexaenoic acid	ω-3 22:6

*All double bonds are in *cis*-configuration

Long-chain polyunsaturated fatty acids (PUFAs) have more than one double carbon bond and 18 or more carbon atoms. They are classified according to the position of the first double bond as counted from the methyl terminus. An ω-3 PUFA has its first double bound at position 3 as counted from the methyl terminus. As a synonym of ω, the symbol n is often used to classify PUFAs.

In biological systems, fatty acids are mostly encountered as components of lipids. Lipids are organic compounds that are insoluble in water and soluble in organic solvents. Chemically, lipids vary to such a great extent that no structural definition is available (Gurr and Harwood 1991).

Two general types of lipids exist; the complex and the simple lipids. The complex lipids, such as for example the triacylglycerols, can be hydrolysed to yield smaller molecules. The simple lipids, including for example the steroids, cannot be hydrolysed into smaller molecules (McMurry 1988). The most familiar lipids, the acyl- and phosphoglycerols, are based on glycerol and fatty acids. The acylglycerols have a glycerol backbone linked to one, two or three fatty acids via ester bonding, yielding mono-, di- and triacylglycerols, respectively. 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 of groups (Gurr and Harwood 1991).

A fat is lipid material that is solid at room temperature whereas an oil is a similar material that is liquid at room temperature. Natural oils and fats consist mainly of triacylglycerols (Fig. 1). For this reason, the terms oil and fat are often used to denote triacylglycerols. However, other components exist that are present in small amounts in natural fats and oils. They include mono- and diacylglycerols, phospholipids, waxes, steroids and carotenoids (Stauffer 1996).

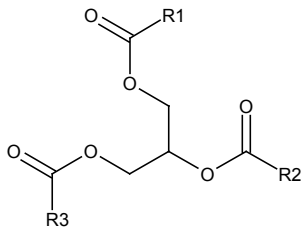

Fig. 1 Schematic representation of triacylglycerol, with in the middle the glycerol backbone and on the outside the fatty acyl groups (R1-CO, R2-CO and R3-CO).

Biological functions of lipids

Lipids are indispensable for growth and survival of all organisms. They are important structural components of membranes and, in many organisms, play a crucial role in carbon and free-energy storage. Evolution has selected triacylglycerols as one of the major storage materials of cells. They have a high free-energy content and a tendency to form aggregates in water, which allows for compact unhydrated intracellular packing (Stryer 1988). In times of plenty, triacylglycerols can be stored and in times of starvation or strong exercise this storage can be used. 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. Microorganisms can store triacylglycerol as intracellular oil droplets. The acylglycerols play no or a little part in membrane structure.

The lipids that contribute to the structure and function of biological membranes are called structural lipids. Widely distributed structural lipids include phospholipids, glycolipids (lipids containing a sugar constituent), sphingolipids and steroids. Structural lipids contain a (long) hydrophobic and a (shorter) hydrophilic part. They can form sheet-like double layers where the hydrophobic and hydrophilic parts are oriented to the inside of the layer and to the external water phase, respectively. 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

The ω-6 PUFA arachidonic acid is the precursor for eicosanoids like thromboxanes, prostaglandins and leukotrienes (Sprecher 1981). Eicosanoids are molecules that are active in regulation of critical biological functions by altering cell activities. The eicosanoids occur and are biologically active in virtually every mammalian tissue (Stanley and Miller 1998). Furthermore, 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 especially the ω-3 PUFAs are 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 1997a). The British Nutrition Foundation recommended a ω-6 to ω-3 PUFA ratio between 5:1 and 3:1 (British Nutrition Foundation 1992).

With respect to infant nutrition, ω-3 PUFAs, and especially docosahexaenoic acid (DHA, Fig. 2), are essential for foetal growth and infant development (Innis 1991). DHA is regarded to be essential for the proper visual and neurological development of infants because of its roles as structural lipid component (Nettleton 1993). Breast feeding serves as a good source of PUFAs. Infant formulas, however, are generally devoid of these fatty acids (Huisman et al. 1996). As infants are unable to synthesise DHA at a rate fast enough to keep up with the demand from the rapidly growing brain (Newton 1998), it has been recommended that all infant formulas include DHA and arachidonic acid (FAO/WHA Expert committee 1994). These PUFAs should also be represented at adequate levels in the diet of breast-feeding mothers.

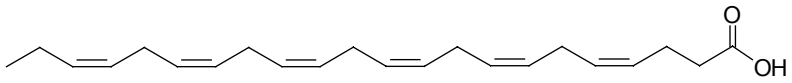

Fig. 2 Schematic representation of docosahexaenoic acid (DHA; ω-3 22:6).

Fatty acid biosynthesis

Chain elongation

In fatty acid synthesis, acetyl-CoA is converted into the fatty acid palmitic acid (16:0) by the fatty acid synthetase system (FAS). The first step in this process (Table 2) is the formation of malonyl-CoA by carboxylation of acetyl-CoA, an ATP-dependent reaction that is catalysed by acetyl-CoA carboxylase. The CO_2 involved in this reaction is again released in a later reaction. In fact, all

carbon atoms of the fatty acids (with an even number of carbon atoms) are derived from acetyl-CoA.

Acetyl transacylase catalyses the conversion of acetyl-CoA and acyl carrier protein (ACP) into acetyl-ACP and CoA (step 2). Malonyl transacylase catalyses the conversion of malonyl-CoA and ACP into malonyl-ACP and CoA (step 3). In four subsequent reactions (steps 4-7) butyryl-ACP, CO_2, ACP, H_2O and 2 $NADP^+$ are formed at the expense of acetyl-ACP, malonyl-ACP and 2 ($NADPH + H^+$). These reactions are catalysed by acyl-malonyl-ACP condensing enzyme, β-ketoacyl-ACP-reductase, 3-hydroxyacyl-ACP-dehydratase and enoyl-ACP reductase, respectively.

Table 2 Principal reactions in fatty acid synthesis (Stryer 1988).

Step	Reaction	Enzyme
1	Acetyl-CoA + HCO_3^- + ATP → malonyl-CoA + ADP + Pi + H^+	Acetyl-CoA carboxylase
2	Acetyl-CoA + ACP ↔ acetyl-ACP + CoA	Acetyl transacylase
3	Malonyl-CoA + ACP ↔ malonyl-ACP + CoA	Malonyl transacylase
4	Acetyl-ACP + malonyl-ACP → acetoacetyl-ACP + ACP + CO_2	Acyl-malonyl-ACP condensing enzyme
5	Acetoacetyl-ACP + NADPH + H^+ ↔ D-3-hydroxybutyryl-ACP + $NADP^+$	β-Ketoacyl-ACP-reductase
6	D-3-hydroxybutyryl-ACP ↔ crotonyl-ACP + H_2O	3-hydroxyacyl-ACP-dehydratase
7	Crotonyl-ACP + NADPH + H^+ → butyryl-ACP + $NADP^+$	Enoyl-ACP reductase

In each next next round of elongation the fatty acyl chain grows with two carbon atoms. The overall stoichiometry of the synthesis of palmitate (16:0) is (Stryer 1988)

$$8 \text{ Acetyl-CoA} + 7 \text{ ATP} + 14 (\text{NADPH}+\text{H}^+) \rightarrow \text{palmitate} + 14 \text{ NADP}^+ + 8 \text{ CoA} + 6 \text{ H}_2\text{O} + 7 \text{ ADP} + 7 \text{ P}_i$$

There are two types of fatty acid synthetases. Type I FAS is present in mammals, birds, yeasts, fungi and some special bacteria. In this type all enzyme activities are located on one or two polypeptide chains (Schweizer et al. 1984; Schweizer 1989 and 1996). 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 (Töpfer and Martini 1994). Palmitate (16:0) formed via the FAS enzyme system can be elongated by steps of 2 carbon atoms (again provided by a malonyl-CoA precursor) to 18:0-24:0 (Schweizer 1989).

Desaturation

Fatty acids with double bonds can be synthesised anaerobically in many prokaryotes, including *Escherichia coli* (Bloch 1969). These double bonds are a consequence of a lack of enoyl reduction after the dehydratation (Hopwood and Sherman 1990) and thus the double bond remains (note that crotonyl-ACP has a double bond at Δ2, Table 2). Eukaryotes, cyanobacteria and some bacilli can introduce double bonds into fatty acids by using oxygen-dependent desaturases (Bloomfield and Bloch 1960; Shanklin and Cahoon 1998). The various desaturases are named according to the position of the double bond they introduce in the fatty acyl chain. For example, a Δ9 desaturase introduces a double bond at position Δ9. Desaturases can be soluble, but most of these enzymes are membrane associated (Bloch 1969; Shanklin and Cahoon 1998). In yeasts, desaturases are three-component enzyme systems consisting of cytochrome b_5 reductase, cytochrome b_5 and the desaturase itself (Fig. 3; Ratledge 2001; Stryer 1988).

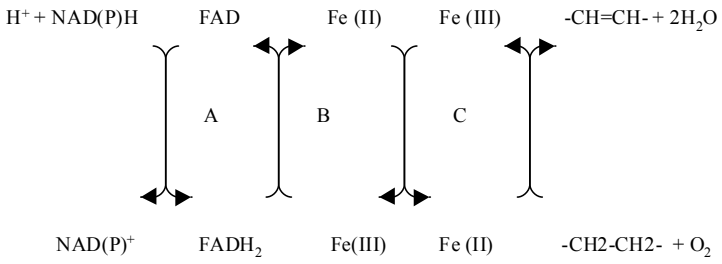

Fig. 3 Mechanism of the desaturases (modified from Ratledge 2001 and Stryer 1988). A= cytochrome b_5 reductase; B= cytochrome b_5; C= fatty acid desaturase. Either NADPH or NADH is used.

The best known desaturases are the Δ9 desaturases, responsible for the conversion of 16:0 to Δ9 16:1 and of 18:0 to Δ9 18:1. In *Saccharomyces cerevisiae,* the *OLE1* gene encoding the Δ9 desaturase is essential for the production of monounsaturated fatty acids (Stukey et al. 1989; Stukey et al. 1990). Furthermore**,** the regulation of its expression has been studied (McDonough et al. 1992; Choi et al. 1996; Gonzalez and Martin 1996). This work has facilitated the isolation and characterisation of Δ9 desaturase genes from several other yeasts (Meesters and Eggink 1996; Meesters et al. 1997; Anamnart et al. 1997).

Various other desaturases have been studied. A few examples are mentioned. Δ12 and Δ6 desaturases from the fungus *Mortierella alpina* were cloned and expressed in the yeast

Saccharomyces cerevisiae. This enabled the yeast to convert Δ9 18:1 into ω-6 18:3, a fatty acid normally not found in *S. cerevisiae* (Huang et al. 1999).

Also from marine microorganisms, several desaturase-encoding genes have been studied. For example, the structural genes encoding the Δ12 (Wada et al. 1990; Sakamoto et al. 1994), the Δ6 (Reddy et al. 1993), and the Δ9 desaturases (Sakamoto and Bryant 1997) from the cyanobacterium *Synechocystis* sp. have all been cloned and characterised. Furthermore, the Δ4 desaturase from the marine organism *Traustochytrium* sp., when expressed in *S. cerevisiae*, introduced a double bond at the Δ4 position of externally supplied ω-3 22:5 and ω-6 22:4. This resulted in the production of DHA and docosapentaenoic acid (ω-6 22:5), respectively (Qiu et al. 2001).

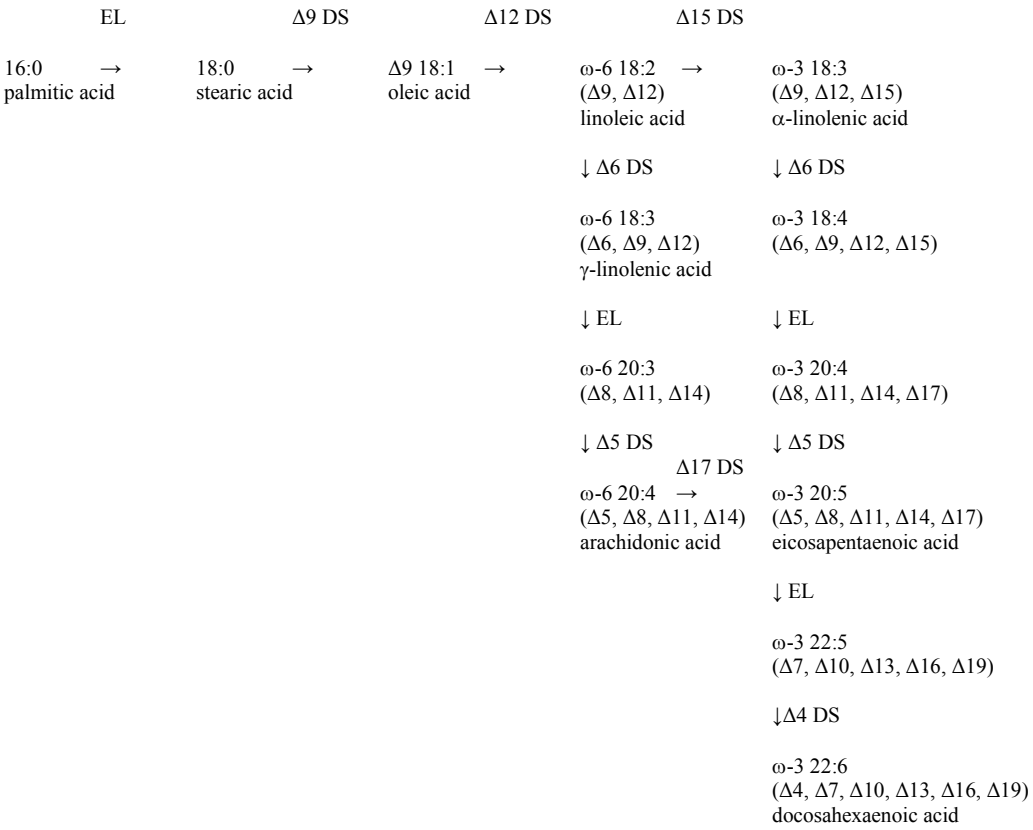

Fig. 4 Routes of ω-6 and ω-3 polyunsaturated fatty acid biosynthesis in fungi (modified from Ratledge 1993). The abbreviations DS and EL indicate reactions catalysed by desaturases and elongases, respectively. The common names of several fatty acids are indicated.

Biosynthesis of PUFAs

Many oil-seed plants produce significant amounts of linoleic acid and no γ-linolenic acid. Linoleic acid could be converted to the nutritionally important γ-linolenic acid by a Δ6 desaturase if it were present. In a first step to produce γ-linolenic acid by oil seed plants the Δ6 desaturase from *Synechocystis* sp was expressed in transgenic tobacco plants. This indeed led to the production of γ-linolenic acid (Reddy and Thomas 1996).

After the synthesis of 16:0 or 18:0 fatty acids by the FAS system, elongation and desaturation reactions can lead to various mono- and polyunsaturated fatty acids. As mentioned above, desaturases can introduce double bonds at various positions in the fatty acyl chains. The biosynthesis of PUFAs requires a cascade of reactions, catalysed by a variety of desaturases and elongases. Although the routes of PUFA biosynthesis in most organisms are not yet completely understood, a generalised overview of current knowledge on ω-6 and ω-3 PUFA biosynthesis in fungi is given in Fig. 4 (Ratledge 1993). In different groups of organisms, the presence, absence and differential regulation of enzyme activities leads to completely different fatty acid profiles.

As an alternative to the fungal pathway shown in Fig. 4, PUFAs can be produced anaerobically in the marine bacterium *Shewanella* sp (Metz et al. 2001). The sequence of reactions of anaerobic PUFA biosynthesis remains to be determined.

Commercial production of non-PUFA lipids

Applications

The average annual production level of 17 commodity oils was over 103 million tonnes in the five-year period 1996-2000 (Table 3). The large majority of this amount was designated for human consumption (80-81%). About 5-6% was used for lifestock feed, thus indirectly contributing to human food production. Finally, the oleochemical industry uses approximately 14% of the annual turnover of commodity oils (Gunstone 2001).

The basic oleochemicals (chemicals derived from oils and fats) are fatty acids, methyl esters, fatty alcohols, fatty amines and glycerol. In volume, surface-active components are the most important applications but other applications are numerous. Some end-user markets include

candles, cosmetics, detergents, food emulsifiers, paints, pharmaceuticals, plastics, soaps and textiles (Gunstone 2001). Some oils have been and are being evaluated for use in biodiesel production (Diesel 1912a and b; Knothe and Dunn 2001).

Table 3 Average annual production (million tonnes) during the five-year period 1996/2000 of 17 commodity oils (Gunstone 2001).

Plant oils	**Amount**	**Animal fats/oils**	**Amount**
Soybean	22.84	Tallow	7.65
Palm	17.93	Lard	6.21
Rape/canola	12.56	Butterfat	5.75
Sunflower	9.14	Fish	1.11
Groundnut	4.62		
Cottonseed	4.00		
Coconut	3.10		
Olive	2.42		
Palm kernel	2.26		
Corn	1.97		
Linseed	0.73		
Sesame	0.70		
Castor	0.47		
Total plant	82.74	Total animal	20.72
Total plant + animal	103.46		

Throughout history, oils and fats have made up an essential part of the human diet. Fats are nutritionally important for various reasons. As mentioned, fat is a dense store of energy. Furthermore, it carries fat-soluble vitamins (A, D and E). The fatty acids in the fats can become part of membrane lipids. Finally, some fats contain PUFAs needed by the body for the reasons discussed above. In food processing, fats and oils can provide structure or lubrication in foods and are used in baking and frying. As fats and oils contribute to flavour and texture they also have sensory functions in foods (Stauffer 1996).

Lipid production from animal and plant materials

Natural sources of oils and fatty acids include plants, animals and microorganisms. Several oils from plants (like olive and sunflower oil) and animals (like butter and lard) are very well known over history, mostly for their applications in nutrition. Currently, plant oils account for the majority of the natural oils and fats on the world market (Table 3).

Before natural fats and oils become edible or applicable they must be processed. The complexity of this process depends on the source and the product requirements. For example, oils from seeds are obtained either by squeezing or by solvent extraction. Further processing may require refining of the oil to remove impurities.

The fats and oils from different sources can differ to a great extent. The primary component of fats and oils, triacylglycerol, varies strongly as the three fatty acids of a specific triacylglycerol molecule are mostly not the same and the oil from a given source often contains a mixture of various triacylglycerols (McMurry 1988). In general, fats and oils from animals predominantly consist of saturated and mono-unsaturated fatty acids. Fish oils, however, contain a high proportion of PUFAs. Plant oils contain a wide variety of fatty acids (Gurr and Harwood 1991).

Plant oils have some advantages over animal fats and oils. Plants are primary producers of oil, using solar light as the energy source. The production methods are relatively cheap. Agricultural methods have been optimised over history and crop selection methods have increased the productivity of plants. Therefore, more plant oils are available than animal oils and fats. Furthermore, plant oils are considered to be healthier due to their fatty acid profile, which contains more unsaturated fatty acids than animal fats (with the notable exception of fish oils).

Microbial production of lipids

As a source of oil or, in more general terms, lipids, microorganisms are less well known than plants and animals. Microbial oil or single cell oil (SCO) production is a relatively new concept, first proposed in the twentieth century (Ratledge 2001). In SCO processes, microorganisms that are able to produce the desired oil are cultivated in a bioreactor.

As the prices for most bulk plant oils are relatively low, and animal fats are even cheaper, it is unlikely that processes for the microbial production of oils resembling these ‘common’ oils can ever be commercially viable. This problem can be illustrated by attempts to produce cocoa butter equivalents by *Cryptococcus curvatus* (Davies 1992) and γ-linolenic acid (GLA) by *Mucor circinelloides* or *Mortierella isabella* (du Preez et al. 1995; Nakahara et al. 1992). The developed processes were optimised and technically feasible. However, in a commercial sense the projects failed because the production costs were too high as compared to the world-market prices of the

product. Although these early attempts to commercially produce SCOs have failed, the concept has now yielded several economic successes and industrial interest is increasing (Barclay et al. 1994; Kyle 1996; Kyle 1997; Ratledge 2001).

Evidently, in order to be able to develop economically feasible oil production processes by cultivation of oleaginous microorganisms in bioreactors it is important that the oils or fatty acids are produced at a high productivity and are of a sufficiently high value. The factors that determine the overall volumetric productivity are the final dry biomass, oil content of the biomass, desired fatty acid of the oil and the process duration. The value of oils and fatty acids are dependent on market mechanisms.

Several PUFAs currently have a good market value, because they cannot be easily produced in bulk quantities from plant or animal materials. However, several very good microbial oil producers (e.g. the yeast *Cryptococcus curvatus*) do not produce the relevant PUFAs. Genetic engineering of these organisms may, in the future, potentially lead to the production of tailor-made oils with high market values. However, at present, oil production by non-engineered organisms is preferred as food ingredients from genetically engineered sources are not widely accepted by consumers. So far, only relatively few wild-type microorganisms able to produce high valued oils at a good rate have been identified. Some of these will be discussed below. Characteristically, their oils contain a high proportion of PUFAs.

Regulation of lipid accumulation in microorganisms

Depending on the microbial species and environmental conditions, the lipid content of microorganisms may vary between a few per cent to over 80% of the biomass dry weight (Ratledge 1993; Leman 1997). To make some kind of a distinction, the term oleaginous has been introduced. Microorganisms are called oleaginous if they are able to accumulate over 20-25% lipid on a dry biomass basis (Ratledge and Evans 1989). Oleaginous microorganisms store lipids mainly in the form of triacylglycerols. Various eukaryotes can accumulate large amounts of triacylglycerols whereas bacteria (with only a few exceptions) do not (Ratledge 1993).

Lipid accumulation is a dynamic process, which depends on the microorganism, the growth conditions (like pH, temperature, nutrients and aeration) and the growth phase. Therefore, the proper selection of the microorganism, process optimisation and timing of harvest are

essential for efficient SCO production. Most oleaginous microorganisms start to accumulate oil whenever excess carbon source is present while, at the same time, growth is limited by another nutrient (in practice often the nitrogen source; Kessell 1968; Ratledge and Evans 1989). Consequently, batch processes for the production on microbial oils typically have separate phases for biomass production and lipid accumulation (Fig. 5).

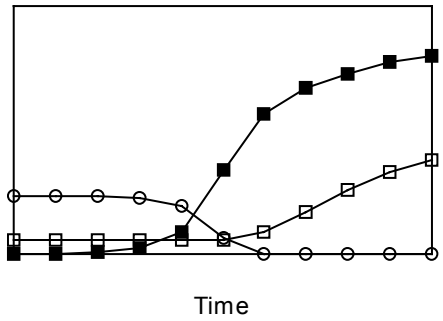

Fig. 5 Idealised representation of lipid accumulation during batch growth of oleaginous microorganisms. In unspecified units, (■) dry biomass, (□) lipid content of dry biomass and (○) nitrogen concentration of the growth medium (modified from Ratledge 2001).

The synthesis of fatty acids is a cytosolic process with acetyl-CoA as the basic building block (Ratledge and Evans 1989). The routes of supply of cytosolic acetyl-CoA depend on the carbon source used for growth and on the organism. Ratledge and Evans (1989) have reviewed possible routes starting with C6-sugars as carbon sources. Simplified, the main flux of carbon from glucose to cytosolic acetyl-CoA in oleaginous yeasts (and probably in other oleaginous eukaryotes) involves glycolysis, transport of pyruvate into the mitochondrion, conversion of pyruvate into citrate, transport of citrate into the cytosol and cleavage of citrate by ATP:citrate lyase to yield acetyl-CoA (Fig. 6). Consistent with this general pathway, all oleaginous yeasts described to date contain ATP:citrate lyase (the enzyme is absent in bakers' yeast, Ratledge and Evans 1989).

In theory, acetyl-CoA may be supplied in the cytosol in a more direct way, namely by growing the organism on C_2-compounds like acetate. The conversion of acetate into acetyl-CoA involves a one-step enzymatic reaction catalysed by the enzyme acetyl-CoA synthetase (Fig. 6). Reports on the localisation of acetyl-CoA synthetase in *Saccharomyces cerevisiae* are conflicting (van den Berg and Steensma 1995). Acetyl-CoA synthetase has been localised in the

mitochondrion, microsomes (Klein and Jahnke 1971) and cytosol (Kispal et al. 1991) of this yeast. Cytosolic activity of acetyl-CoA synthetase has also been detected in mammals (Knudsen et al. 1992), insects (Storey and Bailey 1978) and plants (Gerbling et al. 1994). Therefore, it seems interesting to investigate the applicability of C_2-compounds as carbon sources for lipid accumulation by oleaginous eukaryotes.

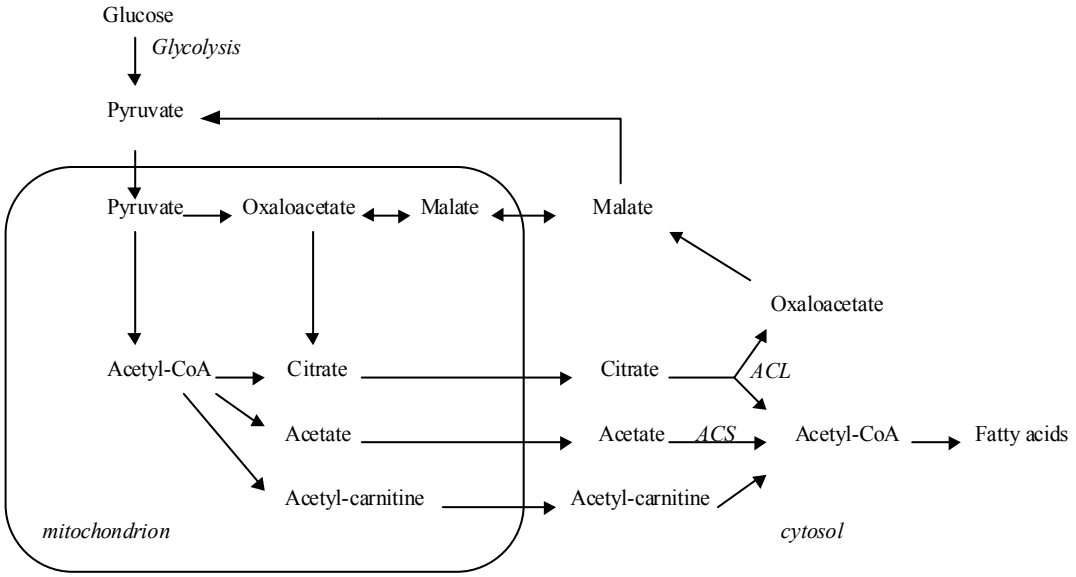

Fig. 6 Acetyl-CoA metabolism in oleaginous yeasts (modified from Ratledge and Evans 1989). ACL=ATP:citrate lyase, ACS=acetyl-CoA synthetase.

Commercial production of PUFAs

Applications

The intake of ω-3 PUFAs via our diet occurs mainly via the consumption of sea food, which is characteristically rich in ω-3 PUFAs. The average intake varies among populations. Intake is high by Greenland eskimos (10-14 g/day), intermediate in countries such as Japan and Norway (1-3 g/day) and low in most Western populations (<0.5 g/day; Schmidt et al. 2001). In an expert panel, there was a general agreement that two fish-based meals per week is a healthy dietary habit to

obtain sufficient ω-3 PUFAs. In practice, this does not often occur in Western diets (Nordøy et al. 2001).

PUFAs are also included in the diet of life stock to raise the PUFA content of their products. For example, eggs and milk enriched with DHA are on the market (Horrocks and Young 1999).

As mentioned before, PUFAs are applied in formulations and pharmaceuticals (Newton 1998). DHA and arachidonic acid are applied in products for pregnant and breast-feeding women and in infant formulas (Kyle 1997; Kyle 1996) to provide developing embryos and babies with sufficient amounts of these fatty acids. Also, fish oil capsules are available for this purpose.

A strong demand for PUFAs results from the introduction of large-scale marine fish farms. The normal growth and development of several marine fish larvae depend on the supplementation of ω-3 PUFAs in the diet, particularly DHA and eicosapentaenoic acid (Rodríguez et al. 1998).

PUFA production from non-microbial resources

Linoleic acid can be obtained from seeds of corn, cotton, safflower and soy, whereas the main sources of γ-linolenic acid are borage (starflower), evening primrose and blackcurrant. Green vegetables such as cabbage, spinach, broccoli and lettuce provide most of the α-linolenic acid in our diet. The main source of longer fatty acids, including DHA and eicosapentaenoic acid, are fatty fish species such as herring, mackerel, sardine and salmon (Gunstone 1996).

The application of PUFAs from fish oils in foods or for inclusion in infant formulas has some disadvantages. They are often unsuitable because of contamination of the fish by environmental pollution and problems associated with the typical fishy smell and unpleasant taste. In addition, fish oils generally contain eicosapentaenoic acid, an undesirable component in infant formulas because it leads to reduced arachidonic acid levels in infants. This has been correlated with reduced rates of infant weight gain (Carlson 1996).

The demand for PUFAs in human nutrition, fish feeds and pharmaceutical applications is rapidly growing. It is therefore expected that, in the near future, the production of purified PUFAs from current sources will become inadequate for supplying the expanding market (Gill and Valivety 1997b). In order to meet the expected rise in demand and to circumvent the drawbacks

of fish oils, alternative production processes for PUFAs are currently being developed. These include the development of refining techniques of fish oils (Yamamura and Shimomura 1997) and the exploitation of microbial PUFA sources (Barclay et al. 1994; Ratledge 2001).

Microbial production of PUFAs

As an alternative to fish oil, PUFAs can be obtained from microorganisms. Microorganisms, in particular the marine algae, are thought to be the primary producers of ω-3 PUFAs in the marine food chain. Although marine fish and mammals appear to have some capacity for *de novo* biosynthesis of ω-3 PUFAs, the majority of the PUFAs in their body originates from their diet (Ackman et al. 1964).

Microorganisms capable of producing PUFAs containing 20 or more carbon atoms include lower fungi, bacteria and marine microalgae (Bajpai et al. 1991; Gunstone et al. 1994; Kendrick and Ratledge 1992). With few exceptions, bacteria are probably not suitable as SCO producers, as they do not accumulate high amounts of triacylglycerols (Barclay et al. 1994; Ratledge 1993).

Oleaginous microorganisms could provide an economical source of PUFAs, provided that most of the PUFAs occur in triacylglycerols which are the preferred species to take up lipids in the diet (Kendrick and Ratledge 1992). Furthermore, microorganisms preferably contain one specific PUFA rather than a mixture of various PUFAs. This gives the microbial oils an additional value as compared to fish oils, which contain mixtures of PUFAs. Furthermore, PUFAs can be purified more easily (and thus more economically) from oils which contain one PUFA instead of a mixture of PUFAs.

The development of a microbial PUFA production process requires the selection of the proper microorganism and optimised cultivation techniques (Ratwan 1991). Several oleaginous fungi are capable of the production of high amounts of PUFAs in their lipid (Kendrick and Ratledge 1992). Several species of *Mortierella* (Table 4) and some other genera of fungi were identified as producers of arachidonic acid (ω-6 20:4). Their oils do not contain ω-3 PUFAs. A selected strain of the fungus *Mortierella alpina* is currently used as a commercial source of oil rich (approximately 40%) in arachidonic acid (ARASCO). The growth medium of *M. alpina* in this process is based on glucose and either yeast extract or hydrolysed vegetable protein. During

cultivation, the temperature, pH, air flow, pressure, agitation, glucose concentration and dissolved oxygen tension are monitored and controlled. In the industrial-scale fermentation, cells are harvested at maximum volumetric arachidonic productivity. Subsequently, the cells are dried and the oil is processed (Kyle 1997).

The exploitation of marine algae for the commercial production of PUFAs will be discussed in the next paragraph.

Table 4 Percentages of specific fatty acids in the lipids of selected microorganisms. References: a, Yamada et al. (1992); b, Yokochi et al. (1998); c, Singh and Ward (1996); d, Molina Grima et al. (1993); e, Servel et al. (1994); f, Viso and Marty (1993); g, Harrington and Holz (1968). P and H indicate phototrophic and heterotrophic growth, respectively. All microorganisms listed in the Table are marine organisms, except for *Mortierella alpina* and *Mortierella elongata*.

Organisms	Growth	14:0	16:0	16:1	18:0	18:1	ω-6 18:2	ω-6 18:3	ω-3 18:4	ω-6 20:4	ω-3 20:5	ω-3 22:6	others
Mortierella alpina CBS 754.68[a]	H		14		7	10	6	5		52			6 (ω-6 20:3)
Mortierella elongata[a] (H)	H		17		8	33	7	7		23			4 (ω-6 20:3)
Schizochytrium limacinum SR21[b]	H	3	49		1						1	33	6 (ω-6 22:5)
Thraustochytrium aureum[c]	H	3	8			16	2	2		3		52	
Isochrysis galbana[d]	P	12	10	11	1	3	2		11		25	11	
Skeletonema costatum[e]	P	17	17	11		2	1		6		41	7	
Amphidinium sp[f]	P	5	27		18	17	2	2			8	17	4 (20:0)
Crypthecodinium cohnii[g]	H	19	20	1	1	14						30	2 (10:0), 8 (12:0)

PUFA production by marine algae

Biotechnological applications of marine algae

Marine microorganisms represent the greatest percentage of undescribed marine species (Colwell 1997). As the marine environment is a rich source of biological and chemical diversity, the marine microorganisms (and other marine species) form a large potential source of commercially interesting compounds. As it may often be impossible to chemically synthesise the discovered compounds, the organisms containing the compounds are at first likely to be the source of the compound. However, harvesting the organisms from their natural environment may lead to environmental damage and allow for insufficient product yields. Alternatively, the compound of interest may be produced by cultivation of the organism. For this, detailed knowledge of the nutritional requirements of the organism and optimisation of fermentation processes are required. Marine microorganisms that have so far been cultivated outside their natural environment include algae, fungi and bacteria.

The biotechnological applications of marine microalgae are very diverse. Several examples are mentioned. Carotenoids, used for coloration of salmon flesh, are produced by microalgae (Margalith 1999). Microalgae are used in beauty masks and shampoos. Phycoerythrin, used in enzyme-linked immunosorbent assays (ELISAs) and for flow cytometry, are produced by cultivation of red algae (Glazer 1989). Microalgae can be used for the production of silica e.g. for filter agents and catalyst supports (Vrieling 1999). Screening efforts of marine microalgae have identified antibiotics and pharmacologically active compounds. Several of these compounds may find applications in pharmaceuticals or in agriculture (Borowitzka 1995). Currently, the production of PUFAs by microalgae (Table 4) is subject of intensive research and increasing commercial attention (Ratledge 2001).

Most algae are phototrophic; they need light as the free-energy source for growth. Alternatively, some algae can be grown heterotrophically. They do not need light as a free-energy source, but derive metabolic energy from the dissimilation of organic carbon compounds.

The oldest and simplest systems for cultivation of phototrophic algae are open ponds. These cultivation systems are dependent on the weather and climate. The product quantity and

quality of separate batches is therefore variable. Processes are time consuming due to the low specific growth rates of algae and available light limits the attainable biomass concentrations. Due to contamination with bacteria and predation by protozoa, phototrophic cultivation is only feasible when suitable selective environments can be used (e.g. high salinity). Furthermore, due to the low biomass concentrations, harvesting costs are relatively high (Barclay et al. 1994).

In closed photobioreactors, the environmental parameters can be better controlled, allowing for higher biomass concentrations and a reduced contamination risk. Scale-up of the process is however limited by the ability to effectively introduce the light (Pulz 2001).

As mentioned, heterotrophic cultivation is independent of light. The production occurs in bioreactors, which can be operated axenically and under controlled optimal conditions. Further advantages include higher biomass concentrations, increased reproducibility and straightforward scale-up of the fermentation processes (Chen 1996).

PUFA production by heterotrophic marine algae

Heterotrophic marine algae used for the production of ω-3 PUFAs include *Traustochitrium* and *Schizochytrium* species (Table 4). These species are sometimes also classified as marine fungi (Henderson 1999). High levels of DHA are also found in dinoflagellates, such as *Crypthecodinium cohnii* and *Amphidinium* sp. (Table 4). Dinoflagellates are early eukaryotic marine algae that can be phototrophic and/or heterotrophic and form an important part of the marine plankton (Gobillard 1996; Sonnenborn and Kunau 1982). Gobillard (1996) has reviewed the peculiar organisation and functioning of the genome of dinoflagellates.

Currently, at least two commercial processes exist for the production of ω-3 PUFAs by heterotrophic cultivation of marine algae (Barclay et al. 1994).

Schizochytrium sp. is used to produce DHA by heterotrophic cultivation (OmegaTech, Boulder, Colorado, USA). A major drawback of *Schizochytrium* strains is the production of ω-6 docosapentaenoic acid (DPA) in the microbial oils, in addition to DHA (Nakahara et al. 1996; Yokochi et al. 1998; Ratledge 2001). The nutraceutical properties of DPA are currently not well known and therefore its presence in oils for food and pharmaceutical applications is undesirable. Separation of DPA from DHA is difficult and expensive.

The dinoflagellate *Crypthecodinium cohnii* has been identified as a good producer of DHA (Table 4). *C. cohnii* can accumulate lipid to over 20% of its biomass dry weight, with a high content of DHA (over 30% of the total lipid). Other PUFAs represent less than 1% of the *C. cohnii*-derived oil (Harrington and Holz 1968; Beach and Holz 1973). DHA is commercially produced by cultivation of *C. cohnii* (Martek Biosciences, Columbia, Maryland, USA).

Crypthecodium cohnii

This thesis focuses on the production of DHA by *Crypthecodinium cohnii*. This chloroplastless heterotrophic marine microalga, formerly known as *Gyrodinium cohnii*, has been studied since the end of the nineteenth century (Seligo 1887). Heterotrophy in algae implies the capacity for sustained growth and cell division in the dark. Free energy and cell carbon are both obtained from the metabolism of an organic substrate. In nature, *C. cohnii* can be found on rotting seaweed. On a medium containing yeast extract, sodium acetate and peptone in sea water, *C. cohnii* can readily be propagated in the laboratory (Pringsheim 1956). The relative ease with which *C. cohnii* could be cultivated as compared to other heterotrophic algae, prompted studies to discover its optimal growth conditions and to design defined media (Beach and Holz 1973; Tuttle and Loeblich 1975).

Two forms of *C. cohnii* exist; swarming cells and cysts. Both forms can vary strongly in size (10-50 μm and beyond). Swarming or swimming cells are motile due to two flagella; a transverse and a longitudinal one. A normal speed of swimming cells is about 1 m h^{-1}. The cysts are either in a resting/survival or dividing stage. Out of one cyst, 1, 2, 4 or 8 swarming daughter cells can originate. The alga was originally believed to be asexual, but later evidence for sexuality was found (Beam and Himes 1974). Various groups (e.g. Bhaud et al. 1991) have studied division and the cell cycle(s) of *C. cohnii* in detail.

Research intensified when its abovementioned characteristics with respect to DHA accumulation became apparent (Harrington and Holz 1968; Beach and Holz 1973). Relative simple and cheap purification protocols suffice to isolate pure DHA from *C. cohnii* oils (Ward 1995). In addition to this, *C. cohnii* appears to be an interesting model organism to study the largely unknown pathways involved in the biosynthesis of PUFAs by marine algae.

Scope of this thesis

The aim of the research described in this thesis was to identify relevant process parameters for the large-scale production of docosahexaenoic acid (DHA) with *Crypthecodinium cohnii.* Several cultivation protocols for *C. cohnii* were developed, analysed and optimised with respect to the production of biomass, lipid and DHA and solutions were sought for newly identified bottlenecks for industrial DHA production.

Chapter 2 describes the development of a simple batch cultivation protocol for *C. cohnii*, using media consisting of glucose, yeast extract and artificial sea salt. The influence of the concentrations of yeast extract, glucose and sea salt on biomass, lipid and DHA production were studied. Furthermore, the impact of different antifoaming agents, carbon sources, temperatures and stirring speeds were investigated. The results were implemented into a protocol for batch cultivation of *C. cohnii* in 2-l bioreactors.

Chapter 3 describes the discovery of novel extracellular polysaccharides in *C. cohnii* cultures. Polysaccharide production became apparent due to the increased viscosity of the culture supernatant during batch cultivation. The polysaccharides were characterised with respect to their chemical and physical characteristics and their influence on culture viscosity and oxygen transfer in bioreactors were determined.

In Chapter 4, fed-batch cultivations of *C. cohnii* on (50% w/v) glucose and (50% w/w) acetic acid as carbon sources were compared. The addition of acetic acid could be controlled by a pH-stat mechanism; the pH was kept constant by addition of acetic acid. The feed rate of glucose was manually controlled. Biomass, lipid and DHA production were much higher in fed-batch cultivations with acetic acid than with glucose as carbon source. By optimisation of the acetic acid protocol, for the first time with *C. cohnii*, high-cell-density cultivation (> 100 g l^{-1} dry weight) was achieved. The increased viscosity of the growth medium due to polysaccharide production by *C. cohnii* resulted in a need for vigorous aeration. The viscosity and thereby the need for such vigorous aeration could be decreased by the addition of a polysaccharide degrading enzyme preparation.

In Chapter 5, experiments are described that were designed to scale-up the acetic-acid fed-batch process to 150-l scale.

The research described in Chapter 6 was designed to evaluate whether ethanol may be an interesting carbon source for fed-batch cultivation and DHA production with *C. cohnii*. Firstly, the sensitivity of *C. cohnii* for ethanol was determined. Secondly, an ethanol-feeding strategy was developed. The dissolved oxygen tension was used to control ethanol toxicity during ethanol feeding. In a partially optimised process with ethanol as carbon source, the productivity of DHA was higher than previously found with glucose and acetic acid as the carbon source.

The pathways of DHA biosynthesis in *C. cohnii* are largely unknown. In Chapter 7, the ability of *C. cohnii* to use ^{13}C labelled externally supplied short- and long-chain carboxylic acids as precursor molecules for DHA biosynthesis was tested. Furthermore, the presence of desaturases (typical for aerobic polyunsaturated fatty acid synthesis) was studied by the addition of desaturase inhibitors in the growth medium of *C. cohnii*.

References

Ackman RG, Jangaard PM, Hoyle RJ, Brockerhoff H (1964) Origin of marine fatty acids. Analysis of the fatty acids produced by the diatom *Skeletonema costatum*. J Fish Res Bd Canada 21:747-756

Anamnart S, Tomita T, Fukui F, Fujimori K, Harashima S, Yamada Y, Oshima Y (1997) The *P-OLE1* gene of *Pichia angusta* encodes a Δ9-fatty acid desaturase and complements the *ole1* mutation of *Saccharomyces cerevisiae*. Gene 184:299-306

Bajpai P, Bapai PK, Ward OP (1991) Production of docosahexaenoic acid by *Traustochytrium aureum*. Appl Microbiol Biotechnol 35:706-710

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6:123-129

Beach DH, Holz GG (1973) Environmental influences on the docosahexaenoate content of the triacylglycerols and phosphatidylcholine of a heterotrophic, marine dinoflagellate, *Crypthecodinium cohnii.* Biochim Biophys Acta 316:56

Beam CA, Himes M (1974) Evidence for sexual fusion and recombination in the dinoflagellate *Crypthecodinium* (*Gyrodinium*) *cohnii*. Nature 250:435-436

Berg MA van den, Steensma HY (1995) *ACS2*, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose. Eur J Biochem 231:704-

713

Bhaud Y, Salmon JM, Soyer-Gobillard MO (1991) The complex cell cycle of the dinoflagellate protoctist *Crypthecodinium cohnii* as studied *in vivo* and by cytofluorimetry. J Cell Science 100:675-682

Bloch K (1969) Enzymatic synthesis of monounsaturated fatty acids. Acc Chem Res 2: 193-202

Bloomfield DK, Bloch K (1960) Formation of Δ9-unsaturated fatty acids. J Biol Chem 235:337-345

Borowitzka MA (1995) Microalgae as sources of pharmaceuticals and other biologically active compounds. J Appl Phycol 7:3-15

British Nutrition Foundation (1992) In: Unsaturated fatty acids, nutritional and physiological significance. The report of the British Nutrition Foundation's task force, Chapman and Hall, London, pp 152

Carlson SE (1996) Arachidonic acid status of human infants: Influence of gestational age at birth and diets with very long chain n-3 and n-6 fatty acids. J Nutr 126:S1092-S1098

Chen F (1996) High cell density culture of microalgae in heterotrophic growth. TIBTECH 14:421-426

Choi JY, Stukey J, Hwang SY, Martin CE (1996) Regulatory elements that control transcription, activation and unsaturated fatty acid-mediated repression of the *Saccharomyces cerevisiae OLE1* gene. J Biol Chem 271:3581-3589

Colwell RR (1997) Microbial diversity and biotechnology. In: Reaka-Kudla ME, Wilson E (eds) Biodiversity II: understanding and protecting our biological resources. Joseph Henry Press, Washington, DC, pp 279-287

Davies RJ (1992) Scale up of yeast technology. In: Kyle DJ, Ratledge C (eds) Industrial applications of single cell oils. American oil chemists' society, Champaign, Illinois, pp 196-218

Diesel R (1912a) The diesel oil-engine. Engineering 93:395-406

Diesel R (1912b) The diesel oil-engine and its industrial importance particularly for Great Britain. Proc Inst Mech Eng:179-280

FAO/WHO Expert committee (1994) Food and nutrition paper no 57. FAO, Rome

Gerbling H, Axiotis S, Douce R (1994) A new acyl-CoA synthetase, located in higher plant cytosol. J Plant Physiol 143:561-564

Gill I, Valivety R (1997a) Polyunsaturated fatty acids, part 1: occurrence, biological activities and applications. TIBTECH 15:401-409

Gill I, Valivety R (1997b) Polyunsaturated fatty acids, part 2: biotransformations and biotechnological applications. TIBTECH 15:470-478

Glazer AN (1989) Light guides. Directional energy transfer in a photosynthetic antenna. J Biol Chem 264:1-4

Gobillard MO (1996) The genome of the primitive eukaryote dinoflagellates: organization and functioning. Zoological studies 35:78-84

Gonzalez CI, Martin CE (1996) Fatty acid-responsive control of mRNA stability: unsaturated fatty acid-induced degradation of the *Saccharomyces ole1* transcript. J Biol Chem 271:25801-25809

Gunstone FD (1996) Fatty acid and lipid chemistry, Blackie Academic, London

Gunstone FD, Harwood JL, Padley FB (1994) The Lipid Handbook. Chapman and Hall, London

Gunstone FD (2001) Basic oleochemicals, oleochemical products and new industrial oils. In: Gunstone FD, Hamilton RJ (eds) Oleochemical manufacture and application. Sheffield Academic Press, Sheffield, pp 1-22

Gurr MI, Harwood JL (1991) Lipid biochemistry, 4th ed. Chapman and Hall, London

Harrington GW, Holz GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochim Biophys Acta 164:137-139

Henderson RJ (1999) The production of n-3 polyunsaturated fatty acids in marine organisms. Lipid technol 11:5-10

Hopwood DA, Sherman DH (1990) Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu Rev Genet 24:37-66

Horrocks LA, Young KY (1999) Docosahexaenoic acid-enriched foods: production and effects on blood lipids. Lipids 34:S313

Huang YS, Chaudhary S, Thurmond JM, Bobik EG Jr, Ling Y, Chan GM, Kirchner SJ, Mukerji P, Knudzon DS (1999) Cloning of Δ12- and Δ6-desaturases from *Mortierella alpina* and recombinant production of γ-linolenic acid in *Saccharomyces cerevisiae*. Lipids 34:649-659

Huisman M, Beusekom CM van, Lanting CI, Nijeboer HJ, Muskiet FAJ, Boersma ER (1996) Triglycerides, fatty acids, sterols, mono- and disaccharides and sugar alcohols in human milk and current types of infant formula milk. Eur J Clin Nutr 50:255-260

Innis SM (1991) Essential fatty acids in growth and development. Prog Lipid Res 30:39-103

Kang JX, Leaf A (1996) The cardiac antiarrhytmic effects of polyunsaturated fatty acid. Lipids 31:S41-S44

Kendrick A, Ratledge C (1992) Lipids of selected molds grown for production of n-3 and n-6 polyunsaturated fatty acids. Lipids 27:15-20

Kessell RHJ (1968) Fatty acids of *Rhodotorula gracilis*: fat production in submerged culture and the particular effect of pH value. J Appl Bacteriol 31:220-231

Kispal G, Cseko J, Alkonyi I, Sandor A (1991) Isolation and characterisation of carnitine acetyltransferase from *Saccharomyces cerevisiae*. Biochim Biophys Acta 1085:217-222

Klein HP, Jahnke L (1971) Variations in the localisation of acetyl-coenzyme A synthetase in aerobic yeast cells. J Bacteriol 106:596-602

Knothe G, Dunne RO (2001) Biofuel derived from vegetable oils and fats. In: Gunstone FD, Hamilton RJ (eds) Oleochemical Manufacture and applications. Sheffield Academic Press, Sheffield, pp 106-163

Knudsen CT, Immerdal L, Grunnet N, Quistorff B (1992) Peritonal zonation of the cytosolic acetyl-CoA synthetase of male rat liver. Eur J Biochem 204:359-362

Kremer JM (1996) Effects of modulation of inflammatory and immune parameters in patients with rheumatic and inflammatory disease receiving dietary supplementation of n-3 and n-6 fatty acids. Lipids 31:S243-247

Kromann N, Green A (1980) Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. Acta Med Scand 208:401-406

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. Lipid Technol 8:107-110

Kyle DJ (1997) Production and use of a single cell oil highly enriched in arachidonic acid. Lipid Technol 9:116-121

Leman J (1997) Oleaginous microorganisms: an assessment of the potential. Adv Appl Microbiol 43:195-243

Margalith PZ (1999) Production of ketocarotenoids by microalgae. Appl Microbiol Biotechnol 51:431-438

McDonough VM, Stukey JE, Martin CE (1992) Specificity of unsaturated fatty acid-regulated expression of the *Saccharomyces cerevisiae OLE1* gene. J Biol Chem 267:5931-5936

McMurry J (1988) Organic chemistry, 2nd ed. Brooks/Cole, Pacific Grove, California

Meesters PAEP, Eggink G (1996) Isolation and characterization of a Δ9 fatty acid desaturase gene from the oleaginous yeast *Cryptococcus curvatus* CBS 570. Yeast 12:723-730

Meesters PAEP, Springer J, Eggink G (1997) Cloning and expression of the Δ9 fatty acid desaturase gene from *Cryptococcus curvatus* ATCC 20509 containing histidine boxes and a cytochrome b_5 domain. Appl Microbiol Biotechnol 47:663-667

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthetases in both prokaryotes and eukaryotes. Science 293:290-293

Molina Grima E, Sánchez Pérez JA, Garciá Camacho F, Garciá Sánchez JL, López Alonso D (1993) n-3 PUFA productivity in chemostat cultures of microalgae. Appl Microbiol Biotechnol 38:599-605

Nakahara T, Yokocki T, Kamisaka Y, Suzuki (1992) γ-Linolenic acid from genus *Mortierella*. In: Kyle DJ, Ratledge C (eds) Industrial applications of single cell oils. American oil chemists' society, Champaign, Illinois, pp 61-97

Nakahara T, Yokochi T, Higashihara T, Tanaka S, Yagishi Y, Honda D (1996) Production of docosahexaenoic and docosapentaenoic acid by *Schizochytrium* sp. isolated from Yap islands. J Am Oil Chem Soc 73:1421-1426

Nettleton JA (1993) Are n-3 fatty acids essential nutrients for fetal and infant development? J Am Dietetic Assoc 93:58-64

Newton IS (1998) Long-chain polyunsaturated fatty acids-the new frontier in nutrition. Lipid technol 10:77-81

Nordøy A, Marchioli R, Arnesen H, Videbæk J (2001) n-3 Polyunsaturated fatty acids and cardiovascular diseases. Lipids 36:S127-129

Preez JC du, Immelman M, Kock JFL, Kilian SG (1995) Production of γ-linolenic acid by *Mucor circinelloides* and *Mucor rouxii* with acetic acid as carbon substrate. Biotechnol letters 17:933-938

Pringsheim EG (1956) Micro-organisms from decaying seaweed. Nature 178, 480-481

Pulz O (2001) Photobioreactors: production systems for phototrophic microorganisms. Appl Microbiol Biotechnol 57: 287-293

Qiu X, Hong H, MacKenzie SL (2001) Identification of a Δ4 fatty acid desaturase from *Thraustochytrium* sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. J Biol Chem 276:31561-31566

Ratledge C, Evans CT (1989) Lipids and their metabolism. In: Rose AH, Harrison JS (eds) The yeasts, 2nd ed. Academic Press, London, pp 367-455

Ratledge C (1993) Single cell oils-have they a biotechnological future? TIBTECH 11:278-284

Ratledge C (2001) Microorganisms as sources of polyunsaturated fatty acids. In: Gunstone FD (ed) Structured and modified lipids. Marcel Dekker, New York, pp 351-399

Ratwan SS (1991) Sources of C_{20}-polyunsaturated fatty acids for biotechnological use. Appl Microbiol Biotechnol 35:421-430

Reddy AS, Nuccio ML, Gross LM, Thomas TL (1993) Isolation of a Δ6-desaturase gene from the cyanobacterium *Synechocystis* sp. strain PCC 6803 by gain-of-function expression in *Anabaena* sp. strain PCC 7120. Plant Mol Biol 22:293-300

Reddy AS, Thomas TL (1996) Expression of cyanobacterial Δ6-desaturase gene results in gamma-linolenic acid production in transgenic plants. Nature Biotechnol 14:639-642

Rodríguez C, Pérez JA, Badía P, Izquierdo MS, Fernández-Palacios H, Hernández L (1998) The n-3 highly unsaturated fatty acids requirements of gilthead seabream (*Sparus aurata* L.) larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture 169:9-23

Sakamoto T, Bryant DA (1997) Temperature-regulated mRNA accumulation and stabilization for fatty acid desaturase genes in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Mol Microbiol 23:1281-1292

Sakamoto T, Wada H, Nishida I, Ohmori M, Murata N (1994) Identification of conserved domains in the Δ12 desaturases of cyanobacteria. Plant Mol Biol 24:643-650

Seligo A (1887) Untersuchungen über Flagellaten. In: Cohn (ed) Beitrage zur Biologie des Pflanzen 4, pp145-180

Schweizer M, Lebert C, Holtke J, Roberts LM, Schweizer E (1984) Molecular cloning of the yeast fatty acid synthetase genes, *FAS1* and *FAS2*: illustrating the structure of the *FAS1* cluster gene by transcript mapping and transformation studies [*Saccharomyces cerevisiae*]. Mol Gen Genet 194:457-465

Schweizer E (1989) Biosynthesis of fatty acids and related compounds. In: Ratledge C,

Wilkinson SG (eds) Microbial lipids 2, Academic Press, London, pp 3-50

Schweizer E (1996) Fatty acid synthase- functional strategies of a multienzyme. Naturwissenschaften 83:347-358

Schmidt EB, Christensen JH, Aardestrup I, Madsen T, Riahi S, Hansen VE, Skou HA (2001) Marine n-3 fatty acids: basic features and background. Lipids 36:S65-68

Servel MO, Claire C, Derrien A, Coiffard L, Roeck-Holtzhauer Y de (1994) Fatty acid composition of some marine microalgae. Phytochemistry 36:691-693

Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol 49:611-641

Singh A, Ward OP (1996) Production of high yields of docosahexaenoic acid by *Traustochytrium roseum* ATCC 20810. J Indust Microbiol 16:370-373

Sprecher H (1981) Biochemistry of essential fatty acids. Prog Lipid Res 20:13-22

Sonnenborn U, Kunay WH (1982) Purification and properties of the fatty acid synthetase complex from the marine dinoflagellate, *Crypthecodinium cohnii*. Biochim Biophys Acta 712:523-534

Sprecher H (1981) Biochemistry of essential fatty acids. Prog Lipid Res 20:13-22

Stanley DW, Miller JS (1998) Eicosanoids in animal reproduction: what can we learn from invertebrates? In: Rowley AF, Kühn H, Schewe T (eds) Eicosanoids and related compounds in plants and animals. Portland Press, London, pp183-196

Stauffer CE (1996) Fats and oils. Eagan Press, St. Paul, Minnesota

Storey KB, Bailey E (1978) Intracellular distribution of enzymes associated with lipogenesis and gluconeogenesis in fat body of the adult cockroach, *Periplaneta*. Insect Biochem 8:125-131

Stryer L (1988) Biochemistry, 3rd ed. WH Freeman and company, New York

Stukey JE, McDonough VM, Martin CE (1989) Isolation and characterisation of *OLE1*, a gene affecting fatty acid desaturation from *Saccharomyces cerevisiae*. J Biol Chem 264:16537-16544

Stukey JE, McDonough VM, Martin CE (1990) The *OLE1* gene of *Saccharomyces cerevisiae* encodes the Δ9 fatty acid desaturase and can be functionally replaced by the rat stearoyl coenzyme A desaturase gene. J Biol Chem 265:20144-20149

Töpfer R, Martini N (1994) Molecular cloning of cDNAs or genes encoding proteins involved in *de novo* fatty acid biosynthesis in plants. J Plant Physiol 143:416-425

Tuttle RC, Loeblich AR (1975) An optimal growth medium for the dinoflagellate *Crypthecodinium cohnii*. Phycologia 14:1-8

Viso AC, Marty JC (1993) Fatty acids from 28 marine microalgae. Phytochemistry 34:1521-1533

Vrieling EG, Beelen TPM, van Santen RA, Gieskes WWC (1999) Diatom silicon biomineralization as an inspirational source of new approaches to silica production. J Biotechnol 70: 39-51

Wada H, Gombos Z, Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature 347:200-203

Ward O (1995) Microbial production of long-chain pufas. INFORM 6:684-688

Yamada H, Shimizu S, Shinmen, Akimoto K, Kawashima H, Jareonkitmongkol S (1992) Production of dihomo-γ-linolenic acid, arachidonoc acid and eicosapentaenoic acid by filamentous fungi. In: Kyle DJ, Ratledge C (eds) Industrial applications of single cell oils. American Oil Chemists' Society, Champaign, Illinois, pp 61-97

Yamamura R, Shimomura Y (1997) Industrial high-performance liquid chromatography purification of docosahexaenoic acid ethyl ester and docosapentaenoic acid ethyl ester from single-cell oil. J Am Oil Chem Soc 74:1435-1440

Yokochi T, Honda D, Higashihara T, Nakahara T (1998) Optimisation of docosahexaenoic acid production by *Schizochytrium limacum* SR21. Appl Microbiol Biotechnol 49:72-76

Chapter 2

Optimisation of docosahexaenoic acid production in batch cultivations of *Crypthecodinium cohnii*

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The heterotrophic microalga *Crypthecodinium cohnii* was cultivated in media containing glucose, yeast extract and sea salt. Increasing amounts of yeast extract stimulated growth but influenced lipid accumulation negatively. Sea salt concentrations above half the average seawater salinity were required for good growth and lipid accumulation. *C. cohnii* was able to grow on a glucose concentration as high as 84 g l^{-1}, although concentrations above 25 g l^{-1} decreased the specific growth rate. Comparison of growth at 27 and 30 ^{o}C showed that the higher incubation temperature was more favourable for growth. However, lipid accumulation was higher at the lower incubation temperature. In a bioreactor the biomass concentration increased from 1.5 to 27.7 g l^{-1} in 74 h. In the final 41 h of the process the lipid content of the biomass increased from 7.5% to 13.5%. In this period, the percentage of docosahexaenoic acid of the lipid increased from 36.5% to 43.6%. The total amounts of lipid and docosahexaenoic acid after 91 h were 3.7 and 1.6 g l^{-1}, respectively.

Introduction

α-Linolenic acid (18:3), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are members of the ω-3 family of fatty acids. In recent years the interest in these long-chain polyunsaturated fatty acids (PUFAs) has increased significantly due to their recognition as being beneficial for human health. They have been reported to play a role in prevention or treatment of a variety of diseases such as arteriosclerosis (Dyerberg 1986; Mehta et al. 1987), thrombosis (Urakaze 1986), arthritis (Kremer et al. 1985) and several types of cancer (Braden et al. 1986; Reddy and Maruyama 1986). In addition, DHA is regarded to be essential for the proper visual and neurological development of infants (Nettleton 1993). DHA is an important PUFA in human breast milk but is generally absent from infant formulas (Huisman 1996). At present, several infant food manufacturers are interested in adding DHA into infant food products (Barclay et al. 1994).

The traditional source of ω-3 fatty acids is fish oil. However, the use of fish oil as a food additive is limited due to problems associated with its typical fishy smell, unpleasant taste, and poor oxidative stability. Furthermore, the presence of EPA in fish oil is undesirable for application in infant food (Carlson 1996). Therefore, alternative sources are of interest.

The heterotrophic microalga *Crypthecodinium cohnii* is an interesting source for DHA production (Kyle et al. 1992; Kyle 1996) and for research on DHA biosynthesis (Beach et al. 1974; Bell and Henderson 1990; Henderson et al. 1988; Henderson and Mackinlay 1991) due to its unique fatty acid composition. *C. cohnii* can accumulate relatively high amounts of lipid (>20%) with 30-50% DHA of the fatty acids and no other polyunsaturated fatty acids present above 1% (Harrington and Holz 1968; Beach and Holz 1973)

So far, a limited number of studies on growth of *C. cohnii* have been reported (Tuttle and Loeblich 1975; Beach and Holz 1973; Vazhappilly and Chen 1998) and the media used were designed for biomass concentrations below 5 g l^{-1}. In this chapter complex media were used consiting of glucose, yeast extract and sea salt. The influence of these medium components and culture conditions on biomass formation, lipid accumulation and DHA content during batch growth of *C. cohnii* was studied in shake flask and bioreactor experiments.

Materials and methods

Chemicals

Yeast extract was obtained from Oxoid. Struktol sb2022 and Struktol j673 were obtained from Struktol. Silicone SE-2 was obtained from Boom (Meppel, The Netherlands). Glucose-monohydrate was obtained from Merck. Sea salt, antifoam 204, docosahexaenoic acid methyl ester (99%), butylated hydroxy toluene (BHT) (>99%) and triheptadecanoic acid glycerate (17:0) (99%) were obtained from Sigma. Methyl docosanoate (22:0) (>99.5%) was obtained from Fluka.

Media, maintenance of cells and cultivation conditions

All medium components were heat-sterilised separately (121 ^{o}C).

C. cohnii (ATCC 30772) cells were grown in static cultures (50 ml in 250 ml shake flasks) on standard medium (9 g l^{-1} glucose, 2 g l^{-1} yeast extract and 27.8 g l^{-1} sea salt) at 25-27 ^{o}C in the dark. The static cultures were subcultured within 4 weeks for maintenance or used within 2 weeks as inoculum for precultures.

Shake flask cultivations (precultures and shake flask experiments), containing 50 ml medium in 250 ml shake flasks, were carried out at 27 ^{o}C, pH 6.5, 100 rpm in a reciprocal shaker. The cultures were inoculated with 10% (v/v) inoculum, unless otherwise indicated. Temperature effects on growth and lipid content were determined at 27 ^{o}C and 30 ^{o}C in duplicate cultures.

Precultures for shake flasks experiments were grown on standard medium and precultures (100 ml in 500 ml shake flasks) for inoculation of batch cultivations in a bioreactor were grown on medium containing 27 g l^{-1} glucose, 3.9 g l^{-1} yeast extract and 27.8 g l^{-1} sea salt. The precultures were incubated for 3-4 days.

In shake flask experiments the cells were harvested after 50 h incubation, unless otherwise indicated.

Batch cultivations were performed at 27 ^{o}C in 2-l laboratory bioreactors (Applikon, Schiedam, The Netherlands). The dissolved oxygen tension was kept above 30% of air saturation by automatically increasing the stirrer speed (starting at 100 rpm) and flushing with 1.0 l min^{-1} filter-sterilised air. The pH was kept constant at 6.5 ± 0.1 by automatic addition of 2 M HCl. The

foam was controlled by automatic addition of 5% (w/v) silicone SE-2. The initial medium (1 l) contained 84 g l^{-1} glucose, 11.5 g l^{-1} yeast extract and 30.6 g l^{-1} sea salt. The inoculum was 20% (v/v), corresponding to 1.5 g l^{-1} biomass.

Determination of biomass concentration and optical density

The biomass concentrations of culture samples (0.5 – 10 ml) were determined using cellulose acetate filters (pore size 0.45 μm, Orange Scientific). After removal of the medium by filtration, the filters were washed with demineralised water and heated at 500 W in a microwave oven for 5 minutes (Samsung electronics, type M 9245).

The optical density (OD) was measured at 470 nm (Pharmacia Biotech, Ultrospec 2000 UV/Visible Spectrophotometer).

Lipid analysis

Culture samples for lipid analysis from shake flask or bioreactor culivations were harvested by centrifugation for 5 minutes at 1,500 *g* and washed once in demineralised water. Prior to and after freeze-drying, the samples were stored at –20 °C.

In order to determine the oil content of small amounts of biomass, the direct transmethylation by a modified procedure of Grayburn et al. (1992) was used. Freeze-dried cells (20-100 mg) were weighed accurately into a Pierce Reacti-Vial and 2 mg triheptadecanoic acid glycerate (17:0) was added as internal standard. Two ml of 1% H_2SO_4 in methanol were added, the vial was vortexed and heated to 80 °C for 2 h. After cooling, 2.0 ml chloroform and 1.0 ml water were added. The vial was vortexed and centrifuged at 1500 *g*. The lower layer containing the fatty acid methyl esters (FAME) was stored at 4 °C prior to gas chromatographic analysis. The amount of lipid was calculated from the total amount of fatty acids and the internal standard. Although this method is useful for determination of the lipid content in small samples, it appeared less useful for DHA analysis due to a relative large variability (Christie 1973). This variability was not observed in the analysis of the other fatty acids present in *C. cohnii*.

For larger amounts of biomass the oil was extracted from freeze-dried cells by a modified method of Bligh and Dyer (1959). Freeze-dried cells (100 mg or more) were weighed accurately

into a 10 ml centrifuge tube. For extraction, 3 ml chloroform:methanol (2:1) containing 1.0 mg ml^{-1} methyl docosanoate (22:0) and 0.5 mg ml^{-1} BHT was used and the tube was shaken gently overnight. After centrifugation at 1,500 *g* for 5 minutes, the supernatant containing the extracted oil was stored at 4 °C until analysis.

Methyl esters of the fatty acids present in this Bligh-Dyer extract were prepared with trimethylsulphonium hydroxide according to Butte (1983).

Fatty acid methyl esters were analysed on a Carlo-Erba GC. A 25 m x 0.25 mm i.d. (d_f = 0.20 µm) Chrompack CP-58 CB column with helium (5.0 quality) as carrier gas. The column temperature was raised from 150 °C to 250 °C with 10 °C min^{-1}, and kept at 250 °C for 10 minutes. One µl samples were injected at a temperature of 275 °C. Detection was performed with a flame ionisation detector (FID) at a temperature of 280 °C. Percentage values of the lipid content of the biomass and the fatty acid composition have been expressed on a weight by weight basis.

For identification purposes GC/MS was performed on a Carlo-Erba Mega GC linked to a QMD-1000 Mass Spectrometer by a direct interface, using the same column and temperature program. Positive ion Electron-Impact Mass Spectra (70 eV) were obtained at a source temperature of 200 °C. The scan range was 25-375 amu. with a scan rate of 1 s^{-1}.

Peaks were identified by comparison of their retention times with authentic references and by comparison of the mass spectra with the spectra present in the Wiley/National Institute of Science and Technology combined library (Wiley Interscience 1994). Values for the lipid content of the biomass and the fatty acids of the lipid are expressed in weight percentages.

Glucose determination

The glucose concentrations in the culture supernatants were determined with the GOD-PAP method (Trinder 1969; Boehringer, Mannheim, Germany).

Results and discussion

Shake flask cultivations

Agitation

Growth of *C. cohnii* was improved significantly by enhancing the agitation speed from 50 to 100 rpm (Fig. 1), probably due to an enhanced oxygen supply. After 50 h growth, the culture grown at 100 rpm had reached a maximum OD of 4.7. This value was more than four times higher than the OD found for the culture grown at 50 rpm. The glucose was entirely depleted after 50 h growth at 100 rpm. This resulted in a decrease of the OD in the following period of incubation. Microscopic examination revealed no detrimental effects of agitation at 50 or 100 rpm which is in contrast with the observations of Tuttle and Loeblich (1975) who found that rotary agitation above 40 rpm in flasks killed the *C. cohnii* cells.

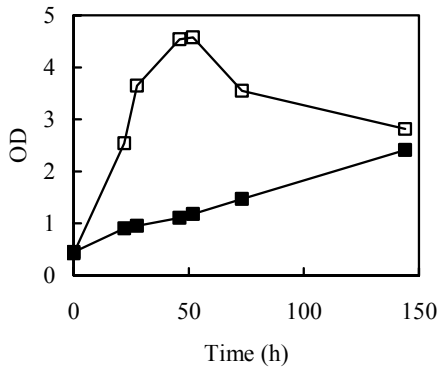

Fig. 1 Growth curves of *C. cohnii* shaken at 50 (■) and 100 (□) rpm.

The importance of aeration conditions for the growth of *C. cohnii* was also indicated by Beach and Holz (1973) who showed that cells with an enhanced supply of O_2, from shake flask and bioreactor cultures multiplied more rapidly than cells grown with a restricted supply of oxygen.

Temperature

The temperature optimum for *C. cohnii* is 27 ^{o}C (Ishida 1968). However, for large-scale cultivations a higher cultivation temperature is desirable, due to an improved cooling capacity at higher temperatures. Therefore, growth and lipid accumulation at incubation temperatures of 27 and 30 ^{o}C were compared.

At 27 and 30 ^{o}C the optical densities of the cultures after 50 h growth were 4.7 and 5.8, respectively. Apparently, growth was more stimulated at the higher incubation temperature.

Also the lipid accumulation was clearly affected by the incubation temperature. When grown at 27 ^{o}C the lipid content was 13.0% compared to 7.8% at 30 ^{o}C. In contrast, Beach and Holz (1973) found a higher lipid content in *C. cohnii* cells grown at 31.5 (11%) than at 25 ^{o}C (8.7%). These authors, however, harvested the cultures grown at 31.5 and 25 ^{o}C at different cultivation times (after four and two days, respectively). This difference in time of harvest may strongly affect the lipid content.

Not only the lipid content but also the percentage of DHA of the lipid was influenced by the incubation temperature. At 27 ^{o}C the percentage of DHA was 35.9% compared to 40.4% at 30 ^{o}C.

Carbon sources

In order to determine suitable carbon sources, growth and lipid accumulation (after 50 h incubation) of *C. cohnii* grown on different carbon sources were studied.

C. cohnii was able to grow on glucose and galactose. After 50 h incubation cultures grown on glucose and galactose had reached comparable OD values of 4.0 and 3.8, respectively. The lipid content of cells grown on glucose was 13.4% whereas cells grown on galactose contained less, 11.4%. The DHA content of the lipids was similar (35.2% with glucose and 36.2% with galactose).

No or marginal growth (less than one doubling) was observed when glycerol and sucrose were offered as carbon sources.

The influence of yeast extract, sea salt and glucose on growth and lipid accumulation

For medium optimisation, the influence of variable amounts of glucose, yeast extract and sea salt on growth and total lipid content of *C. cohnii* was studied.

C. cohnii was cultivated in a medium composed of 9 g l^{-1} glucose, 27.8 g l^{-1} sea salt and a range of yeast extract concentrations (0-10 g l^{-1}). With increasing yeast extract concentrations the resulting OD values after 50 h growth increased (Fig. 2A). The lipid content of the cells, however, decreased markedly at higher yeast extract concentrations. At a yeast extract concentration of 1 g l^{-1}, for example, an OD of 3.8 was reached, whereas the lipid content of the biomass was 20%. At a 5 g l^{-1} yeast extract concentration, the OD increased 1.5 times to 5.8, but the lipid content was only 6%.

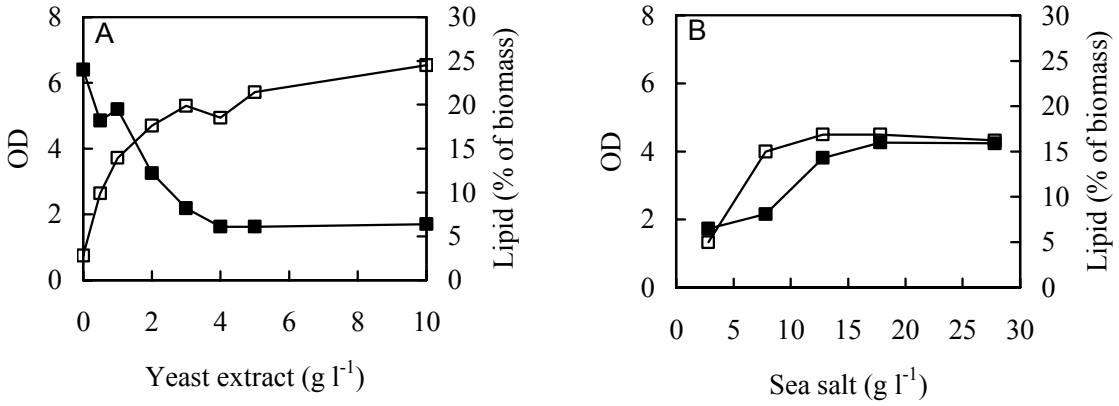

Fig. 2 Effect of different yeast extract and sea salt concentrations on OD (□) and the percentage of lipid of the biomass (■) after 50 h of incubation.

A) *C. cohnii* was grown on media containing 9 g l^{-1} glucose, 27.8 g l^{-1} sea salt and 0-10 g l^{-1} yeast extract.

B) *C. cohnii* was grown on media containing 9 g l^{-1} glucose, 2 g l^{-1} yeast extract and 2.8-27.8 g l^{-1} sea salt.

These results are in agreement with the literature as in general lipid accumulation in microorganisms is stimulated by an excess of a carbon source and a limitation in one of the other nutrients, especially nitrogen (Leman 1997).

The effect of sea salt was studied in media containing 9 g l^{-1} glucose, 2 g l^{-1} yeast extract and sea salt concentrations, ranging from 2.8 to 27.8 g l^{-1}. Both growth and lipid accumulation were stimulated by increasing salinity. At 2.8 and 17.8 g l^{-1} sea salt the OD values after 50 h

growth were 1.8 and 4.2 and the amounts of accumulated lipid were 6 and 16%, respectively (Fig. 2B). A higher salinity (27.8 g l^{-1}) as compared to 17.8 g l^{-1} resulted in similar growth and lipid accumulation. Therefore, for optimal growth and lipid accumulation a minimal sea salt concentration of 17.8 g l^{-1} is required which is about half of the average sea water salinity. The observation of growth inhibition at low salinity is in agreement with previous data. Inhibition of growth at low (<5 g l^{-1} NaCl) and also high salinity (>50 g l^{-1} NaCl) has been reported in shake flask (Beach and Holz 1973) and static (Tuttle and Loeblich 1975) cultivations of *C. cohnii*. For large-scale cultivation processes, the sea salt concentration should preferably be as low as possible in order to prevent corrosion problems.

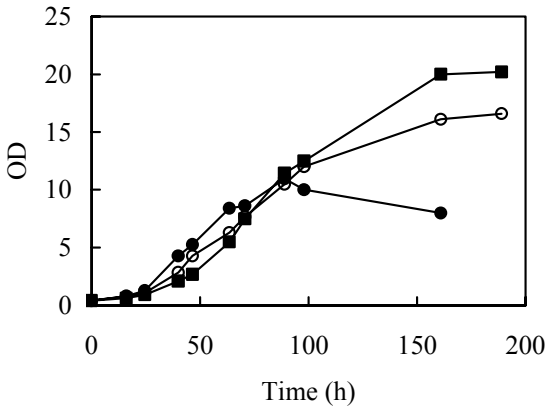

Fig. 3 Effect of different glucose concentrations on growth of *C. cohnii*. Initial medium: 5 g l^{-1} yeast extract, 17.8 g l^{-1} sea salt and glucose; 25 (●), 50 (○) and 75 (■) g l^{-1}.

To study growth on high initial glucose concentrations, *C. cohnii* was incubated with 25, 50 or 75 g l^{-1} glucose in the presence of 5 g l^{-1} yeast extract and 17.8 g l^{-1} sea salt. The highest OD values were reached with the highest glucose concentration (Fig. 3). The initial growth rate, however, decreased at concentrations above 25 g l^{-1} glucose. Average doubling times between 16 and 40 h growth on 25, 50 and 75 g l^{-1} glucose were 10, 12.5 and 13.8 h, respectively. This inhibitory effect of high glucose concentrations on growth of *C. cohnii* has not been described before and is important for further development of batch and fed-batch cultivation methods for the production of DHA by *C. cohnii*.

The influence of different antifoaming agents on growth

In order to study the influence of antifoaming agents on growth of *C. cohnii* four types of antifoam (antifoam 204, Struktol sb2022, Struktol j673 and silicone SE-2) were added to the standard medium at a concentration of 50 μg l^{-1}. In shake flask cultivations only silicone SE-2 did not show detrimental effects on growth and lipid content. Therefore, this agent was selected as a suitable antifoam for bioreactor cultivations.

Bioreactor cultivations

Initial cultivations of *C. cohnii* in a 2-l bioreactor showed that cells were able to grow at a stirrer speed as high as 800 rpm. Furthermore, the doubling time of *C. cohnii,* when grown on standard medium in a bioreactor, was 8.4 h. This doubling time does not significantly differ from the one measured in shake flask experiments (8.7 h) with standard medium.

In order to study the possibility of high cell density cultivation of *C. cohnii* in a batch cultivation and to follow the process of lipid accumulation and fatty acid composition during a growth cycle, a cultivation in a bioreactor was performed.

Exponential growth was observed within the first 43 h of incubation. The average doubling time in the exponential growth phase was 13.3 h, which is similar to the doubling time of 13.8 h found for shake flask experiments with 75 g l^{-1} glucose. After 43 h, the growth rate slowly decreased until growth completely ceased after about 74 h (Fig. 4). At this time the glucose was not entirely depleted. The maximal biomass concentration obtained was 27.7 g l^{-1}, which is the highest biomass concentration described so far for a batch cultivation of *C. cohnii* (Beach and Holz 1973; Kyle et al. 1992). Although the biomass concentration did not further increase after 74 h growth, the glucose concentration continued to decrease, from 16.3 to 8.6 g l^{-1} in the final 17 h.

The lipid content increased from 7.5% at 50 h, to a maximum value of 13.5% at 91 h. This pattern of lipid accumulation fits the general theory that microbial lipid accumulation is a biphasic process (Leman 1997). In the first phase rapid cell division occurs until a nutritional component becomes limiting. In the second phase, the growth rate decreases and lipids start to accumulate.

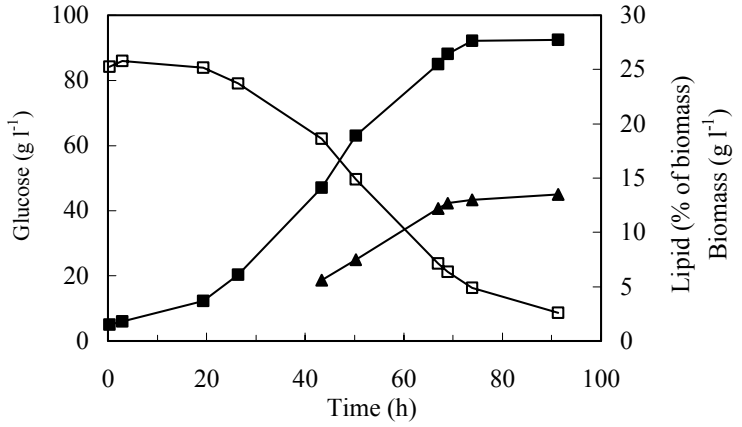

Fig. 4 Growth and lipid content of *C. cohnii* grown in a 2-l bioreactor. Glucose (□), biomass (■) and the percentage of lipid of biomass (▲). Initial medium (1 l): 84 g l^{-1} glucose, 11.5 g l^{-1} yeast extract and 30.6 g l^{-1} sea salt.

DHA (22:6) was the major fatty acid in *C. cohnii* during the final 41 h of incubation (36.5-43.6%) (Table 1). Also abundant were the saturated fatty acids 16:0 (16.9-19.9%), 14:0 (16.6-18.6%), 12:0 (4.6-5.7%) and the monounsaturated fatty acid 18:1 (9.6-10.1%). The fatty acids 10:0, 16:1 and 18:0 were present in smaller amounts (<2.4%).

Table 1 Major fatty acid composition of the total lipid content in percentages of *C. cohnii* grown in a 2-l bioreactor at 50, 67, 74 and 91 h growth. Fatty acid are indicated as y:x where y represents the number of carbon atoms and x the number of double bonds.

Time (h)	10:0	12:0	14:0	16:0	16:1	18:0	18:1	22:6
50	1.5	5.7	16.8	19.9	0.5	2.3	10.1	36.5
67	1	5.7	18.6	18.5	1.3	1.6	9.9	38.3
74	1	5.4	17.6	17.0	1.3	1.5	9.6	41.1
91	0.8	4.6	16.6	16.9	1.3	1.6	9.6	43.6

Interestingly, the percentage of DHA steadily increased from 36.5 to 43.6% between 50 and 91 h. The percentages of other fatty acids remained at a constant level or decreased slightly. This pattern of DHA accumulation in the total lipid fraction was not observed in the study of Beach and Holz (1973). In their data the total fatty acid composition was not mentioned but the fatty acid composition of both triglycerides and phosphatidylcholine (which accounted for 60% of the total lipid) were similar after 1, 2 and 4 days of growth.

The maximal amounts of lipid and DHA (at 91 h) were 3.7 g l^{-1} and 1.6 g l^{-1}, respectively. The average lipid and DHA productivities (at 74 h) were 46 mg l^{-1} h^{-1} and 19 mg l^{-1} h^{-1}, respectively.

In order to produce DHA in a cost-effective process, research should especially focus on an increase of volumetric productivity. Rough cost estimations (Sijtsma et al. 1998) showed that in biocultivations with a relatively low productivity, the fixed costs are the most important cost factor of the total costs per unit of product. An increase in productivity would decrease the relative contribution of these fixed costs and therefore significantly decrease the total product costs. The productivity can be increased by increasing the final biomass concentration, the lipid content of the biomass and the DHA content of the lipid or by decreasing the total process time. This study showed that at least two improvements of the production process of DHA by *C. cohnii* are possible. Firstly, from shake flask cultivations it was concluded that *C. cohnii* cells can contain at least 20% lipid. Such percentages should also be possible in a bioreactor. Secondly, the total process time can be decreased. In a fed-batch cultivation for example, the glucose concentration can be kept below a critical value thereby preventing inhibition of growth.

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References

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6:123-129

Beach DH, Harrington GW, Gellerman JL, Schlenk H, Holz GG (1974) Biosynthesis of oleic acid and docosahexaenoic acid by a heterotrophic marine dinoflagellate *Crypthecodinium cohnii*. Biochim Biophys Acta 369:16-24

Beach DH, Holz GG (1973) Environmental influences on the docosahexaenoate content of the triacylglycerols and phosphatidylcholine of a heterotrophic, marine dinoflagellate, *Crypthecodinium cohnii.* Biochim Biophys Acta 316:56

Bell MV, Henderson RJ (1990) Molecular species composition of phosphatidylcholine from *Crypthecodinium cohnii* in relation to growth temperature. Lipids 25:115-118

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911

Braden LM, Carroll KK (1986) Dietary polyunsaturated fat in relation to mammary carcinogenesis in rats. Lipids 21:285-288

Butte W (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxyde for transesterification. J Chromatogr 261:142-145

Christie WW (1973) Lipid analysis. Pergamon Press, Oxford, pp 85-102

Carlson SE (1996) Arachidonic acid status of human infants: Influence of gestational age at birth and diets with very long chain n-3 and n-6 fatty acids. J Nutr 126:1092-1098

Dyerberg J (1986) Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. Nut Rev 44:125-134

Grayburn WS, Collins GB, Hildebrand DF (1992) Fatty acid alteration by a Δ-9 desaturase in transgenic tobacco tissue. Bio/Technology 10:675-678

Harrington GW, Holz GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochem Biophys Acta 164:137-139

Henderson RJ, Leftley JW, Sargent JR (1988) Lipid composition and biosynthesis in the marine dinoflagellate *Crypthecodinium cohnii*. Phytochemistry 27:1679-1683

Henderson RJ, Mackinlay EE (1991) Polyunsaturated fatty acid metabolism in the marine dinoflagellate *Crypthecodinium cohnii*. Phytochemistry 30:1781-1787

Huisman M (1996) Effects of early infant nutrition and perinatal exposure to PCBs and dioxins on neurological development. PhD thesis, Rijksuniversiteit Groningen, Groningen, The Netherlands

Ishida Y (1968) Physiological studies on evolution of dimethyl sulfide from unicellular marine algae. Mem Res Inst Food Sci Kyoto Univ 94:48-82

Kremer JM, Bigauaoette J, Michalek AV, Timchalk MA, Lininger L, Rynes RI, Huyck C, Zieminsky J, Bartholomew (1985) Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. Lancet:184-187

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. Lipid technol 8:107-110

Kyle DJ, Sicotte VJ, Singer JJ, Reeb SE (1992) Bioproduction of docosahexaenoic acid (DHA) by microalgae. In: Kyle DJ, Radledge C (eds) Industrial Applications of Single Cell Oils. American Oil Chemists' Society, Champaign, Illinois, pp. 287-300

Leman J (1997) Oleaginous microorganisms: an assessment of the potential. Adv Appl Microbiol 43:195-243

Mehta J, Lopez LM, Wargovich T (1987) Eicosapentaenoic acid: its relevance in atherosclerosis and coronary artery disease. Am J Cardiol 59:155-159

Nettleton JA (1993) Are n-3 fatty acids essential nutrients for fetal and infant development? J Am Dietetic Assoc 93:58-64

Reddy BS, Maruyama H (1986) Effect of dietary fish oil on azoxymethane-induced colon carcinogenesis in male F344 rats. Cancer Res 46:3367-3370

Sijtsma L, Springer J, Meesters PAEP, Swaaf ME de, Eggink G (1998) Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae. Recent Res Devel in Microbiology 2:219-232

Trinder P (1969) Determination of glucose in blood using glucose oxidase with alternative oxygen acceptor. Ann Clin Biochem 6:24

Tuttle RC, Loeblich AR (1975) An optimal growth medium for the dinoflagellate *Crypthecodinium cohnii*. Phycologia 14:1-8

Urakaze M, Hamazaki T, Soda Y, Miyamota M, Ibuki F, Yano S, Kumagai A (1986) Infusion of emulsified trieicosapentaenoyl-glycerol into rabbits—the effects on platelet aggregation, polymorphonuclear leukocyte adhesion, and fatty acid composition in plasma and platelet phospholipids. Thromb Res 44:673-682

Vazhappilly R, Chen F (1998) Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth. J Am Oil Chem Soc 75:393-397

Wiley-Interscience (1994) Wiley/NIST combined Library, 6th edn. Wiley-Interscience, New York

Chapter 3

Characterisation of extracellular polysaccharides produced by *Crypthecodinium cohnii*

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The valuable polyunsaturated fatty acid docosahexaenoic acid can be produced by cultivation of the heterotrophic microalga *Crypthecodinium cohnii*. During batch growth of *C. cohnii* on glucose, sea salt and yeast extract for 5 days, so far unreported extracellular polysaccharides were produced. These caused an increased viscosity and a strong drop in the maximum oxygen transfer rate. The viscosity increased most markedly as cells entered the stationary phase. The polysaccharides varied in size (from 6 to >1,660 kDa) and monomer distribution. A high molecular mass fraction (from 100 to >1,660 kDa) and a medium molecular mass fraction (from 6 to 48 kDa) were prepared. The high molecular mass fraction contained (on a molar basis) 71.7% glucose, 13.1% galactose and 3.8% mannose, whereas the medium molecular mass fraction contained 37.7% glucose, 19.8% galactose and 28.1% mannose. Other monomers present in both fractions were fucose, uronic acid and xylose. Monomers were coupled mainly via α-(1-3) links. Increased viscosity due to polysaccharide production complicate the development of commercial high cell density processes for the production of docosahexaenoic acid.

Introduction

The interest in ω-3 long-chain polyunsaturated fatty acids (PUFAs) is growing, due to increasing evidence for their beneficial influence on human health. Roles for PUFAs have been reported in the prevention or treatment of various diseases, e.g. thrombosis (Urakaze et al. 1986) and types of cancer (Braden and Caroll 1986). Furthermore, a sufficient intake of PUFAs by unborn and young children appears to be important for proper visual and neurological development (Nettleton 1993; Hornstra 2000).

The traditional source of PUFAs is fish oil. However, this source is limited and the quality of the oil is variable. Alternative sources are being studied for PUFAs, especially docosahexaenoic acid (DHA, 22:6) which can be used as a functional food ingredient, e.g. for infant nutrition (Barclay et al. 1994). The heterotrophic marine microalga, *Crypthecodinium cohnii*, is particularly known for its ability to accumulate oil with high amounts (30-50%) of DHA with no other PUFAs above 1% (Harrington and Holz 1968).

For economically feasible industrial cultivations of *C. cohnii*, high cell densities are required. Although *C. cohnii* is being used as a commercial source of DHA (Kyle 1996), papers dealing with biomass concentrations above 5 g l^{-1} cell dry weight are very limited. Recently, we described lipid and DHA production by *C. cohnii* in batch cultivations (de Swaaf et al. 1999). It was observed in these experiments that, when cultures reached biomasses above 10-15 g l^{-1} cell dry weight the viscosity of the broth markedly increased. In fermentation processes with non-filamentous microorganisms, increases in the viscosity of the growth medium are due to the production of extracellular polysaccharides (EPSs; Becker et al. 1998). A high viscosity of culture broth increases the energy input required for mixing and may reduce the maximum rate of oxygen transfer. For example, this has been demonstrated in xanthan-producing *Xanthomonas campestris* cultures (Shu and Yang 1990). Moreover, EPS production proceeds at the expense of product formation.

EPS production by *C. cohnii* has so far not been reported. In view of its potential impact on industrial-scale DHA production, we investigated both the production and composition of EPS by *C. cohnii* cultures and their impact on viscosity and oxygen transfer.

Materials and methods

Cultivations

C. cohnii (ATCC 30772) cells were maintained by monthly sub-cultivation in medium (pH 6.4-6.6) recommended by the culture collection, containing: 23.48 g l^{-1} NaCl; 10.63 g l^{-1} $MgCl_2 \cdot 6H_2O$; 3.92 g l^{-1} Na_2SO_4; 1.11 g l^{-1} $CaCl_2$; 0.66 g l^{-1} KCl; 0.19 g l^{-1} $NaHCO_3$; 0.1 g l^{-1} KBr; 0.03 g l^{-1} H_3BO_3; 0.04 g l^{-1} $SrCl_2 \cdot 6H_2O$; 0.012 g l^{-1} $FeCl_3 \cdot 6H_2O$; 0.15 g l^{-1} sodium glycerophosphate; 0.05 g l^{-1} $(NH_4)_2SO_4$; 3.0 g l^{-1} TRIS; 0.01 g l^{-1} K_2HPO_4; 3.0 g l^{-1} glucose; 1.5 g l^{-1} glutamic acid; 0.03 g l^{-1} EDTA; 0.03 g l^{-1} H_3BO_3; 4.5 mg l^{-1} $MnCl_2 \cdot 4H_2O$; 0.3 mg l^{-1} $ZnCl_2$; 0.15 mg l^{-1} $CoCl_2 \cdot 6H_2O$; 0.003 mg l^{-1} biotin and 1 mg l^{-1} thiamine.

Cells were kept static at 25 ^oC. The inoculum size was 10% (v/v) in all cultivations. Media were heat-sterilised (121 ^oC, 20 min) with yeast extract (Oxoid), glucose and sea salt (Sigma) treated separately. Static-grown cultures of 4-10 days old were used to inoculate 50 ml medium (containing: 2 g l^{-1} yeast extract; 25 g l^{-1} sea salt and 9 g l^{-1} glucose) in 300 ml erlenmeyer flasks. These flasks were kept for 2 days in a reciprocal shaker (100 rpm, 27 ^oC) and were used to inoculate 100 ml medium (containing: 5.5 g l^{-1} yeast extract; 25 g l^{-1} sea salt and 25 g l^{-1} glucose) in 500 ml erlenmeyer flasks. After 3 days incubation in a reciprocal shaker, the resulting cells were used to inoculate bioreactors.

Batch cultivations were performed at 27 ^oC in 2-l laboratory bioreactors (Applikon, Schiedam, The Netherlands). The dissolved oxygen tension was kept above 30% of air saturation by automatically controlling the stirrer speed (minimum 100 rpm, maximum 900 rpm) and sparging with filter-sterilised air at 1 l min^{-1}. The pH was kept at 6.5±0.1 by automatic addition of 2 M HCl. Foam production was repressed by automatic addition of 5% (w/v) silicone SE-2 (Boom). The initial media (1 l) contained 7.5 or 15 g l^{-1} yeast extract; 25 g l^{-1} sea salt; 75 g l^{-1} glucose and 10% inoculum. After 5 days cultivation, cells and supernatant were separated by centrifugation (17,000 *g*, 10 min).

Biomass analysis, glucose and viscosity measurements

Culture samples of 20-40 ml were centrifuged (1500 *g*, 5 min). The cell pellet was washed in 25 ml demineralised water and was then lyophilised and weighed, in order to determine the biomass concentration. The lipids from these lyophilised samples were extracted with organic solvents. Methylated fatty acids were prepared and analysed by gas chromatography, as previously described (de Swaaf et al. 1999).

The glucose concentrations in the culture supernatants were determined using the GOD-PAP method (Trinder 1969; Boehringer, Mannheim, Germany). Culture viscosity was determined with a capillary Cannon-Fenske routine viscometer at 27 ^{o}C, according to instructions from the manufacturer (Schott, Mainz, Germany). The media components did not significantly influence the viscosity at the concentrations used.

Oxygen transfer coefficient determination

Values of the oxygen transfer coefficient (k_lA) were determined in a 2-l bioreactor at 27 ^{o}C, using the dynamic method (van 't Riet and Tramper 1991). Two solutions (500 ml) were used: (1) the supernatant of a batch cultivation of *C. cohnii* grown on medium containing 15 g l^{-1} yeast extract; 25 g l^{-1} sea salt; 75 g l^{-1} glucose and 10% inoculum for 5 days and (2) a solution of 25 g l^{-1} sea salt. After sparging the solutions with nitrogen in order to decrease the dissolved oxygen tension below 2%, the solutions were aerated via the headspace (5 l min^{-1}) and stirred (300 rpm). The k_lA values were calculated from plots of the dissolved oxygen tension versus time.

Isolation and purification of crude EPS

Crude EPS from the culture supernatant of a batch cultivation of *C. cohnii* grown on a medium containing 7.5 g l^{-1} yeast extract; 25 g l^{-1} sea salt; 75 g l^{-1} glucose and 10% inoculum for 5 days were isolated as described by Grobben et al. (1997) and lyophilised.

Crude EPS was dissolved in 0.4 M sodium acetate (pH 3.0) to a concentration of 7 g l^{-1}. After removal of insoluble parts by centrifugation, the solution was applied onto a Sepharose 4B column (60 cm; Pharmacia, Roosendaal, The Netherlands) in a fast performance liquid

chromatography system (Pharmacia, Roosendaal, The Netherlands). Elution was performed at a flow rate of 0.5 ml min^{-1}, using 0.4 M acetic acid/sodium acetate (pH 3.0). Samples (2.0 ml) were collected and tested for carbohydrates using the phenol-sulfuric acid method of Dubois et al. (1956). Carbohydrate-containing samples 17-33 (fraction I) and 37-45 (fraction II) were pooled, dialysed and lyophilised.

Non-carbohydrate components of crude EPS

The water and ash content was determined using a Thermal analysis system (7 Series; Perkin-Elmer, Norwalk, Connecticut, USA). The weight of a sample (initially 5 mg) of crude EPS was monitored during heating from 25 ^{o}C to 900 ^{o}C at a rate of 10 ^{o}C min^{-1}.

Capillary electrophoresis was used to determine the Cl content and induced coupled-plasma optical emission spectroscopy (Groen Agro Control, Delft, The Netherlands) was used to quantify the elements S, P, K, Na, Ca, Mg, Si, Fe, Mn, Zn, B, Cu, Co and Mo in the crude EPS solution (2.8 g l^{-1}). The AccQ-Tag method (Waters, Milford, USA) was used to determine the amino acid composition and content.

Molecular mass determinations

High-performance size-exclusion chromatography (HPSEC) was performed using a high-performance liquid chromatography (HPLC) system as described by Stolle-Smits et al. (1999). The system was calibrated using linear pullulans with molecular masses ranging from 6 to 1,660 kDa. Since the EPS produced by *C. cohnii* most probably have a conformation differing from that of pullulans, thereby affecting the elution behaviour, all molecular masses given in this chapter should be regarded as "apparent" values.

Analysis of monosaccharide composition

Samples (3 mg) of crude EPS and partially purified fractions were hydrolysed by being stirred for 2 h at 120 ^{o}C in 1 ml 2 M trifluoroacetic acid (TFA) solution, dried under N_2 at 40 ^{o}C, washed with 0.5 ml 1 M NH_4OH solution, dried again under N_2 and dissolved in 1 ml milli-Q water. The

neutralised hydrolysates were analysed for neutral sugars by using a HPLC system as described by Stolle-Smits et al. (1995). Anhydro-uronic acids of the neutralised hydrolysates were determined as described by Ahmed and Labavitch (1977). The total amount of sugars in crude EPS was used to quantify the amount of EPS.

After hydrolysis of the crude EPS, some material had precipitated. In order to quantify the amount of this precipitate, crude EPS (100 mg) was heated for 2 h at 120 ^{o}C in 50 ml 2M TFA. The precipitate was centrifuged for 10 min at 1,500 *g*, washed, lyophilised and weighed.

Sugar linkage analysis of crude EPS was performed by methylation analysis, according to Ciucanu and Kerek (1984), with an extra sample-pretreatment. The samples were ultrasonically treated in dimethylsulfoxide for 2 h to reach complete dissolution. Subsequent acetylation was performed according to Harris et al. (1984).

The samples were quantified on a gas chromatograph with flame ionisation detection (GC-FID) and identified on a gas chromatograph with mass spectrometric detection (GC-MS). *Myo*-inositol hexaacetate was used as an internal standard.

The GC-FID and GC-MS (both Interscience, Breda, The Netherlands) were equipped with a CP-WAX 58 (FFAP) CB (25m x 0.25mm x 0.2μm) column with helium as carrier gas. Samples of 1 μl were injected at 250 °C. The temperature started at 50 °C and was raised at 10 °C min^{-1} to 250 °C where it was kept for 15 min. The FID was kept at 280 °C and the MS-interface was kept at 250 °C. Mass spectra were recorded at 200 °C and 70 eV using a scan range of 35-510 amu and a rate of 1 scan s^{-1}. The obtained mass spectra were compared to the Wiley/National Institute of Science and Technology combined library (Wiley Interscience 1994).

Orientation of the anomeric protons

The orientation of the anomeric protons (α- or β-orientation) was determined by ^{1}H-NMR experiments on a Bruker AMX400-wb spectrometer (Billerica, USA). The spectra from 10 mg crude EPS in 1 ml D_2O were recorded at 343K, using a 90-degrees radio frequency pulse and a repetition time of 2 s. The data set contained 64,000 data points; and 128 averages were measured.

Precipitation analysis

Precipitation of the crude EPS was studied in the presence of salts and high pH. In cuvettes, 0.5 ml crude EPS (2.6 g l^{-1}), 0.25 ml salt solution (40 mM) and 0.25 ml demineralised water or 0.25 ml NaOH (4 M) were added and vortexed. The salts NaCl, KCl, $CaCl_2$, $MgCl_2$, K_2SO_4, $CuCl_2$, $FeCl_2$, $FeCl_3$, $ZnCl_2$ and $MnCl_2$ were used. For each salt solution, a control was included without the crude EPS. After 1 min incubation the pH was neutralised with 1 ml 1M HCl. Then the cuvettes were vortexed and centrifuged for 3 min. The samples were visually inspected for pellets.

Results

Viscosity

Culture viscosity and EPS production were studied in glucose-grown batch cultures. During 120 h growth of *C. cohnii* on medium containing 75 g l^{-1} glucose; 25 g l^{-1} sea salt; 7.5 g l^{-1} yeast extract and 10% inoculum, glucose was consumed completely (Fig. 1) and 18.3 g l^{-1} biomass was produced. Furthermore, on a per litre supernatant basis, 2.7 g crude EPS were obtained, containing 1.5 g polysaccharides. The viscosity of the medium increased from 0.5 to 3.5 mm^2 s^{-1} and was linearly proportional to the crude EPS concentration (Fig. 2). Therefore, the viscosity of the supernatant can be used indirectly as a measure for the amount of the viscous component of crude EPS.

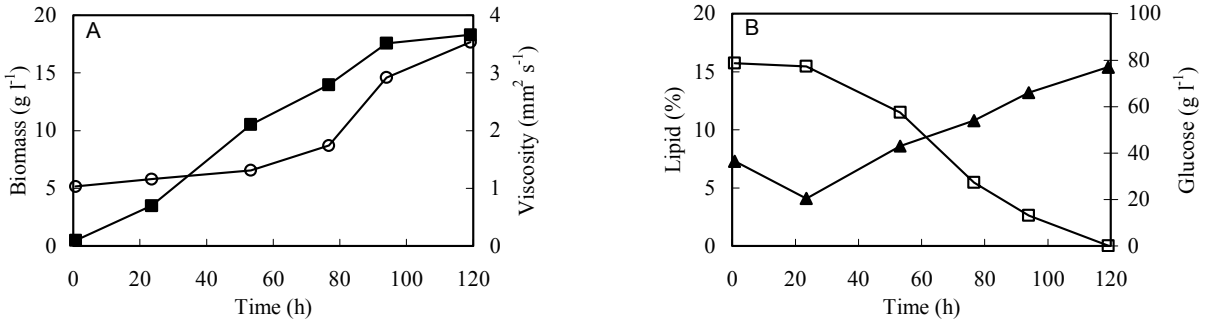

Fig. 1A, B Batch growth of *C. cohnii* on 7.5 g l^{-1} yeast extract, 75 g l^{-1} glucose and 25 g l^{-1} sea salt. A: ■ biomass (g l^{-1}), ○ viscosity (mm^2 s^{-1}). B: ▲ lipid (%), □ glucose (g l^{-1}).

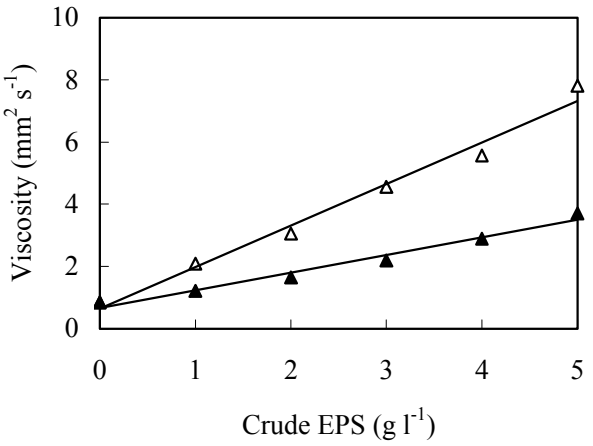

Fig. 2 Relationship between the viscosity and the concentration of crude EPS in demineralised water (Δ) and 25 g l^{-1} sea salt solution (▲).

The production of the viscous component was related to the growth and the growth phase. The viscous component was produced during the entire growth cycle but not at a constant rate. A relatively small amount was produced during the first 53 h, while the biomass reached more than half of its final value. The major part of the viscous component evolved towards the end of the growth phase (at 77-120 h).

In a previous study (de Swaaf et al. 1999), the lipid content of the cells was positively influenced by decreasing the yeast extract concentrations in the medium, while keeping the glucose concentration constant. In order to study the influence of yeast extract on the production of the viscous component, duplicate cultivations on 7.5 and 15 g l^{-1} yeast extract were compared after 120 h growth. By doubling the yeast extract concentration, the biomass increased from 17.1 to 21.4 g l^{-1}. As expected, the lipid content of the cells decreased from 13.8 to 5.1% and the viscosity of the supernatant increased from 4.0 to 5.6 mm^2 s^{-1} (Table 1).

Table 1 Comparison of batch growth of *C. cohnii* on 7.5 and 15 g l^{-1} yeast extract (duplicate cultivations). In both situations, the glucose and sea salt concentrations were 75 g l^{-1} and 25 g l^{-1}, respectively.

Yeast extract (g l^{-1})	Biomass (g l^{-1})	Viscosity (mm^2 s^{-1})	Lipid content of biomass (%)	DHA content of lipid (%)
7.5	17.1± 1.3	4.0 ± 0.5	13.8 ± 1.7	41.5 ± 0.5
15	21.4 ± 1.0	5.6 ± 1.0	5.1 ± 1.5	44.7 ± 1.7

As viscosity may strongly affect oxygen transfer in a bioreactor, values for the oxygen transfer coefficient k_LA were determined. The k_LA value using the supernatant of a batch cultivation grown for 5 days was 3.8×10^{-4} s^{-1}. The k_LA value of the reference solution (25 g l^{-1} sea salt) was 9.5×10^{-4} s^{-1}.

Chemical composition and molecular mass distribution

The crude EPS contained 55% polysaccharides, 10% proteinaceous components, 10% unknown material (precipitate after hydrolysis), 7% ash and trace amounts of Si, Cl, Fe, Zn and Cu. The recovery was 82% (assuming the various components can be summed). Phosphorous and sulfur were not detected. Therefore, the crude EPS contained no or very small amounts of DNA and RNA. Furthermore, fatty acids were not detected.

The molecular masses of the EPS produced by *C. cohnii* varied to a large extent (Fig. 3). The eluted peaks using HPSEC corresponded to molecular masses of >1,660, 1,660, 380, 48 and <6 kDa. The proteinaceous components were only of low molecular mass, indicating that these were not linked to polysaccharides.

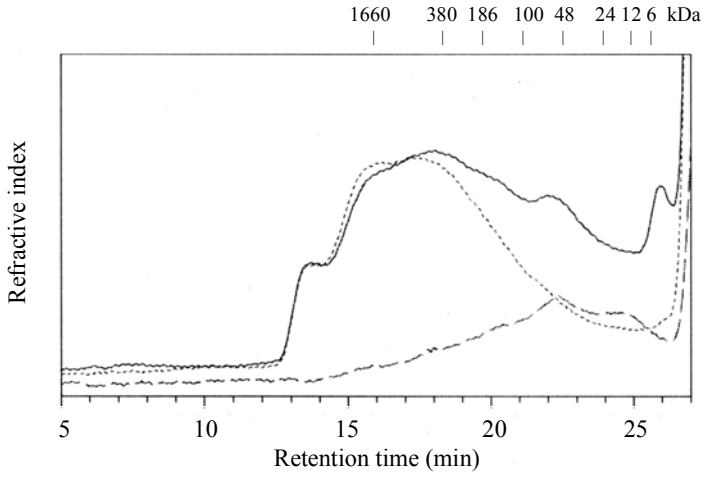

Fig. 3 High performance size-exclusion chromatography elution patterns of crude EPS (line), fraction I (dots) and fraction II (dashes). Indicated are the retention times of linear pullulans with molecular masses ranging from 6 to 1,660 kDa.

Carbohydrate composition

To investigate whether the EPS varied not only in size but also in composition, the crude EPS was fractionated into two fractions and used for the analysis of sugar monomers. Fraction I mainly contained large molecules of 100 to <1,660 kDa whereas fraction II contained molecules ranging from 6 to 48 kDa (Fig. 3). Fraction I was the major fraction; and the total weight of fraction II was 20% of fraction I.

Table 2 Carbohydrate composition (in mol%) of crude extracellular polysaccharides (crude EPS), fraction I and II.

	crude EPS	Fraction I	Fraction II
Glucose	57.7	71.6	37.5
Galactose	17.2	13.1	19.7
Mannose	11.6	3.8	28.0
Fucose	4.6	3.9	4.9
Xylose	1.3	0.2	0.8
Uronic acid	5.6	6.0	7.2
Unknown	2.0	1.3	1.8

Table 2 shows molar percentages of the various carbohydrates in the crude EPS and fractions I and II. The monomers glucose, galactose, mannose, fucose, xylose, uronic acid and an unknown component (<2%) were present in all three samples.

The percentages of the most abundant monomers (glucose, galactose and mannose) in fractions I and II varied to a large extent. Fraction I contained a relative large amount of glucose (71.6%) and galactose (13.1%) and less mannose (3.8%) whereas in fraction II these sugars represented 37.5, 19.7 and 28.0%, respectively. Clearly, *C. cohnii* produced multiple EPSs, variable in size and sugar composition.

Methylation analysis of crude EPS was performed to determine the linkages of sugar residues. From the detected compounds in the GC-MS spectra, 78% could be identified as carbohydrates. The residues were expressed in molar percentages. The EPS consisted mainly of (1-3)-linked glucosyl/mannosyl residues (69%). Other residues were (1-3)-linked galactosyl (3%), (1-4)-linked mannosyl/galactosyl (7%) and (1-6)-linked glucosyl (1%). Furthermore,

branched residues were present to a low extent, namely (1-3-4)-linked mannosyl (3%), (1-2-3)-linked mannosyl/galactosyl (2%) and (1-3-6)-linked glucosyl/mannosyl (2%). Finally, 1-linked end groups glucosyl (9%), galactosyl (2%), xylosyl (2%) and fucosyl (2%) were detected.

^{1}H-NMR spectroscopy was used to determine whether the anomeric protons of the EPS present in crude EPS had mainly α- or β-orientations. The ^{1}H-NMR spectrum of crude EPS showed broad peaks and various anomeric proton resonances, indicating that the EPS are long and heterogeneous (data not shown). Most of the anomeric proton resonance(s) were 5.00-5.60 ppm, indicating that the EPS mainly consisted of α-residues. Minor anomeric signals indicative for β-residues were also present in the spectrum.

Effect of ions

Viscosity of crude EPS depended not only on its concentration, but also on the presence of sea salt. The viscosity was decreased by sea salt (25 g l^{-1}; Fig. 2). Dissolved crude EPS precipitated at high pH in the presence of sea salt. This precipitate did not dissolve at neutral pH. The presence of sea salt was essential for precipitation to occur. To study which ions contributed to this precipitation, several metal ions were incubated with crude EPS at high and neutral pH. A combination of crude EPS, high pH (>12) and one of the cations Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+} or Fe^{3+} resulted in an irreversible precipitation. For this precipitation to occur, both high pH and crude EPS were required. The solubility of the precipitate was not restored after the pH was made neutral. Precipitation of the crude EPS at pH>12 did not occur in the presence of Na^{+}, K^{+}, SO_4^{2-} or Zn^{2+}.

Discussion

Economic feasibility calculations showed that the production costs of DHA by industrial-scale cultivation of *C. cohnii* largely depend on the overall volumetric productivity (Sijtsma et al. 1998). For a high overall volumetric productivity of DHA, high cell densities are a prerequisite. This demands a good oxygen transfer. This chapter shows that the production of (so far unknown) polysaccharides increased the culture viscosity and complicated a good oxygen transfer rate by strongly decreasing the oxygen transfer coefficient (k_LA).

The drop in k_La is especially relevant during (large-scale) industrial cultivations, because large-scale bioreactors generally have a lower maximal k_La than laboratory-scale bioreactors. In industrial cultivations with *C. cohnii*, it can be anticipated that the maximum oxygen transfer rate will decrease in two ways: as a consequence of scaling-up and EPS production. It is likely that the oxygen demand of *C. cohnii* cannot be met during the entire process, even with maximum stirring, aeration and pressure. Consequently, this oxygen limitation reduces the overall volumetric productivity of DHA.

Several other problems associated with the polysaccharide production exist. Yield loss occurs because part of the carbon source is used for polysaccharide production. Furthermore, downstream processing is affected, e.g. we observed that filtration of the cells was difficult due to the viscosity of the culture. Finally, due to the increased viscosity and a need for vigorous stirring, heat production could become problematic in large-scale cultivations.

Evidently, the yeast extract concentration is very important for optimisation of DHA with *C. cohnii*. Growth media with a high carbon-to-nitrogen ratio stimulate microbial lipid accumulation (Leman 1999) and polysaccharide production (Becker et al. 1998). In accordance with de Swaaf et al. (1999), lipid accumulation in *C. cohnii* was strongly decreased by doubling the amount of yeast extract in the cultivation medium. The double amount of yeast extract only slightly stimulated EPS production. The higher amount of EPS may be caused by the higher biomass production. Lipid accumulation and polysaccharide production by *C. cohnii* appear to be triggered differently.

Ideally, in a DHA production process EPS formation by *C. cohnii* should be prevented. This appears difficult to establish, as polysaccharide production was related to both biomass and growth phase. Mutants or other *C. cohnii* strains unable to produce polysaccharides may prove helpful in DHA production processes.

Preliminary analysis showed that the EPS produced by *C. cohnii* was a complex mixture. A large variation of molecular masses was found from <6 to >1,660 kDa. Most molecules were between 48 and 1,660 kDa, corresponding to EPS of 300-10,000 residues. Two fractions separated on the basis of their molecular masses varied strongly in the distribution of glucose, galactose and mannose. Minor residues like uronic acid, fucose and xylose were more conserved. The distribution of the residues from both fractions did not indicate a repeating unit. Likewise, well known marine algal polysaccharides, like alginate, carrageenan and agar vary within and

between samples in subunit composition, arrangement and molecular mass. The arrangement of residues in these polysaccharides is neither completely regular nor completely random (Vreeland et al. 1987).

The EPS consisted mainly of linear (1-3)-coupled residues. The overall branching percentage was 7%. This means that a branching point occured on average every 14 residues. The various residues were mostly in α-configuration. A small amount of residues might have been in β-configuration. The EPS produced by *C. cohnii* appeared unique, due to the variety of sugars and the presence of mostly α-D-(1-3)-hexopyranosyl residues, which are not regularly found in algae.

The polysaccharides could protect cells against dehydration or provide adhesion capacity. The *C. cohnii* cells are known to be adhesive as cysts (Kubai and Ris 1969). Furthermore, polysaccharides may interact with specific compounds for the availability of, e.g. trace elements (ions), or may protect against harmful compounds. Three indications were found for interaction of ions and crude EPS most probably related to the presence of (charged) uronic acids in the EPS. Firstly, crude EPS irreversibly precipitated at a pH > 12 in the presence of several divalent and trivalent cations. Secondly, binding capacity of the crude EPS was evident for the elements Si, Cl, Fe, Zn and Cu. Thirdly, the viscosity of 5 g l^{-1} crude EPS was decreased by using 25 g l^{-1} sea salt as compared to demineralised water.

EPSs are side products of DHA production processes with *C. cohnii* but might be of commercial interest. Applications for EPSs may be found in (functional) food products or in pharmaceuticals. Polysaccharides can have immunological activity, e.g. β-D-(1-3)-glucans can inhibit tumor growth and stimulate the immune system (Bohn and BeMiller 1995). Further studies are required to establish whether the EPS of *C. cohnii* are similarly applicable in foods and/or pharmaceuticals.

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References

Ahmed AER, Labavich JM (1977) A simplified method for accurate determination of cell wall uronide content. J Food Biochem 1:361-365

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6:123-129

Becker A, Katzen F, Pühler A, Ielpi L (1998) Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Appl Microbiol Biotechnol 50:145-152

Bohn JA, BeMiller JN (1995) (1→3)-β-D-glucans as biological response modifiers: a review of structure-functional activity relationships. Carbohydr Polym 28:3-14

Braden LM, Carroll KK (1986) Dietary polyunsaturated fat in relation to mammary carcinogenesis in rats. Lipids 21:285-288

Ciucanu I, Kerek FA (1984) A simple and rapid method for the permethylation of carbohydrates. Carbohydr Res 131:209-217

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350-356

Grobben GJ, Casteren WHM van, Schols HA, Oosterveld A, Sala G, Smith MR, Sikkema J, Bont JAM de (1997) Analysis of the exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. Appl Microbiol Biotechnol 48:516-521

Harrington GW, Holz GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochem Biophys Acta 164:137-139

Harris PJ, Henry RJ, Blakeney AB, Stone BA (1984) An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. Carbohydr Res 127:59-73

Hornstra G (2000) Essential fatty acids in mothers and their neonates. Am J Clin Nutr 71:1262-1269

Kubai DF, Ris H (1969) Division in the dinoflagellate *Gyrodinium cohnii* (Schiller). A new type of nuclear reproduction. J Cell Biol 40:508-528

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. Lipid technol 8:107-110

Leman J (1997) Oleaginous microorganisms: an assessment of the potential. Adv Appl Microbiol 43:195-243

Nettleton JA (1993) Are n-3 fatty acids essential nutrients for fetal and infant development? J Am Diet Assoc 93:58-64

Riet K van 't, Tramper J (1991) Basic Bioreactor Design. Dekker, New York, pp 258-261

Shu CH, Yang ST (1990) Effects of temperature on cell growth and xanthan production in batch cultures of *Xanthomonas campestris*. Biotechnol Bioeng 35:454-468

Sijtsma L, Springer J, Meesters PAEP, Swaaf ME de, Eggink G (1998) Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae. Recent Res Dev Microbiol 2:219-232

Stolle-Smits T, Beekhuizen JG, Dijk C van, Voragen AGJ, Recourt K (1995) Cell wall dissolution during industrial processing of green beans (*Phaseolus vulgaris* L). J Agric Food Chem 43:2480-2486

Stolle-Smits T, Beekhuizen JG, Kok MTC, Pijnenburg M, Recourt K, Derksen J, Voragen AGJ (1999) Changes in cell wall polysaccharides of green bean pods during development. Plant physiol 121:363-372

Swaaf ME de, Rijk TC de, Eggink G, Sijtsma L (1999) Optimisation of docosahexaenoic acid production in batch cultivations by *Crypthecodinium cohnii*. J Biotechnol 70:185-192

Trinder P (1969) Determination of glucose in blood using glucose oxidase with alternative oxygen acceptor. Ann Clin Biochem 6:24

Urakaze M, Hamazaki T, Soda Y, Miyamota M, Ibuki F, Yano S, Kumagai A (1986) Infusion of emulsified trieicosapentaenoyl-glycerol into rabbits - the effects on platelet aggregation, polymorphonuclear leukocyte adhesion, and fatty acid composition in plasma and platelet phospholipids. Thromb Res 44:673-682

Vreeland V, Zablackis E, Doboszewski B, Laetsch WM (1987) Hydrobiologica 151/152 (12th International Seaweed Symposium) Dr Junk Publishers, Dordrecht

Wiley-Interscience (1994) Wiley/NIST combined Library, 6th edn. Wiley-Interscience, New York

Chapter 4

High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*

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The heterotrophic marine alga *Crypthecodinium cohnii* is known to produce docosahexaenoic acid (DHA), a polyunsaturated fatty acid with food and pharmaceutical applications, during batch cultivation on complex media containing sea salt, yeast extract and glucose. In the present study, fed-batch cultivation was studied as an alternative fermentation strategy for DHA production. Glucose and acetic acid were compared as carbon sources. For both substrates, the feed rate was adapted to the maximum specific consumption rate of *C. cohnii.* In glucose-grown cultures, this was done by maintaining a significant glucose concentration (between 5-20 g l^{-1}) throughout fermentation. In acetic acid-grown cultures, the medium feed was automatically controlled via the culture pH. A feed consisting of acetic acid (50% w/w) resulted in a higher overall volumetric productivity of DHA (r_{DHA}) than a feed consisting of 50% (w/v) glucose (38 and 14 mg l^{-1} h^{-1}, respectively). The r_{DHA} was further increased to 48 mg l^{-1} h^{-1} using a feed consisting of pure acetic acid. The latter fermentation strategy resulted in final concentrations of 109 g l^{-1} dry biomass, 61 g l^{-1} lipid and 19 g l^{-1} DHA. These are the highest biomass, lipid and DHA concentrations reported to date for a heterotrophic alga. Vigorous mixing was required to sustain aerobic conditions during high-cell-density cultivation. This was complicated by culture viscosity, which resulted from the production of viscous extracellular polysaccharides. These may present a problem for large-scale industrial production of DHA. Addition of a commercial polysaccharide-hydrolase preparation decreased the viscosity of the culture and the required stirring.

Introduction

The ω-3 long-chain polyunsaturated fatty acids (PUFAs) are valuable ingredients of food and pharmaceutical products due to their beneficial influence on human health. As sufficient PUFA intake by pre-term and young children stimulates visual and neurological development (Nettleton 1993; Hornstra 2000), PUFAs are currently included in various infant foods (Barclay et al. 1994). The traditional source of PUFAs, fish oil, is a limited resource and its composition and quality are variable. Alternative sources of PUFAs, and in particular docosahexaenoic acid (DHA, 22:6), are under investigation.

The heterotrophic marine microalga *Crypthecodinium cohnii* is known for its ability to accumulate lipids with a high fraction (30-50%) of DHA. Other PUFAs account for less than 1% of the total lipid content (Harrington and Holz 1968). Cultivation scale and volumetric productivity (r_{DHA}) have been identified as major factors in determining the economic feasibility of fermentative DHA production (Sijtsma et al. 1998). Factors that determine r_{DHA} are biomass concentration, lipid content of the cells, DHA content of the lipid and cultivation time. Obviously, a high DHA content of the biomass is also desirable from the viewpoint of product recovery.

Current DHA production processes that are based on *C. cohnii* use glucose as the main carbon source (Kyle et al. 1996). Previously, in 90 h glucose-grown batch cultures of *C. cohnii*, we achieved a biomass concentration of 28 g l^{-1} dry weight, with total lipid and DHA contents of 14% and 7%, respectively (de Swaaf et al. 1999).

Acetic acid may be an interesting alternative carbon source for commercial DHA production with *C. cohnii*. In eukaryotes, acetic acid can directly be converted into acetyl-CoA, a key intermediate in lipid synthesis, by acetyl-Coenzyme A synthetase (Woodward and Merrett 1975; Martinez-Blanco et al. 1992; van den Berg et al. 1996). Moreover, its addition to microbial cultures can be easily controlled by a pH-stat mechanism. This fermentation mechanism has been successfully applied for fed-batch cultivations of the gamma-linolenic acid (GLA) producing fungus *Mucor circinelloides* on acetic acid (du Preez et al. 1995). Recent experiments indicate that this procedure is also applicable to DHA production by *C. cohnii* (Ratledge et al. 2001a).

The aims of the present study were to compare glucose and acetic acid as carbon sources for production of DHA in fed-batch cultures of *C. cohnii* and to investigate whether a high-cell-

density fed-batch process for DHA production can be developed with acetic acid as the carbon source. As *C. cohnii* is an obligately aerobic organism, cultivation at high cell densities requires an efficient transfer of oxygen from the gas phase to the culture broth. Recently we demonstrated that *C. cohnii* produces extracellular polysaccharides, which increase culture viscosity, thereby strongly increasing the power input required for mixing and oxygen transfer (de Swaaf et al. 2001). Therefore, we also investigated whether addition of a polysaccharide hydrolase decreases culture viscosity, hence facilitating high-cell-density cultivation of *C. cohnii*.

Elements of this study have been included in a recent patent application (Ratledge et al. 2001b).

Materials and methods

Strain and maintenance

Crypthecodinium cohnii ATCC 30772 was maintained by sub-cultivation on a complex medium containing 2 g l^{-1} yeast extract (Oxoid, Basingstoke, UK); 9 g l^{-1} glucose and 25 g l^{-1} sea salt (Sigma-Aldrich, Zwijndrecht, The Netherlands). Cultures were incubated statically at 25 ^{o}C. The inoculum size in all cultivations was 10% (v/v). All medium components were heat-sterilised separately (121 ^{o}C, 20 min) unless indicated otherwise.

Shake-flask cultivation

Static cultures were grown for 4-10 days and then used to inoculate 50 ml of medium (2 g l^{-1} yeast extract; 25 g l^{-1} sea salt and 9 g l^{-1} glucose) in 300 ml erlenmeyer flasks. These flasks were incubated for 2 days in a reciprocal shaker (100 rpm, 27 ^{o}C) and were subsequently used to inoculate 100 ml of medium (5.5 g l^{-1} yeast extract; 25 g l^{-1} sea salt and 25 g l^{-1} glucose) in 500 ml erlenmeyer flasks. After incubation in a reciprocal shaker for 3 days, these cultures were used to inoculate bioreactors (see below).

Cultivation in bioreactors

Fed-batch cultivations were performed at 27 ^{o}C in 2-l laboratory bioreactors (Applikon, Schiedam, The Netherlands). The condenser was maintained at a temperature of 17 ^{o}C. The dissolved oxygen tension was kept between 30 and 35% of air saturation by automatically controlling the stirrer speed (range: 200-1250 rpm) and by flushing (1 l min^{-1}) with filter-sterilised air. Foam production in high-cell-density cultivations with pure acetic acid as the feed was suppressed by addition of 3-6 droplets of MAZU DF 8005 antifoam (BASF, Cheadle, UK). Foam production in the other cultivations was suppressed with 5% (w/v) silicone SE-2 (Boom, Meppel, The Netherlands), controlled via a foam sensor. The initial medium (1 l) of a fed-batch cultivation with glucose as carbon source, contained 10 g l^{-1} yeast extract; 25 g l^{-1} sea salt; 5 g l^{-1} glucose and a 10% (v/v) inoculum. During the first 24 h of cultivation, a 50% (w/v) glucose solution was fed at a rate of 0.64 g h^{-1} glucose. During the rest of the cultivation the glucose concentration in the medium was kept between 5 and 20 g l^{-1}. To ensure these levels were maintained, samples for glucose determination were taken twice daily and the pump rate was manually adjusted when necessary. The pH was kept at 6.5 ± 0.1 by automated addition of 2M HCl.

In fed-batch cultivations with acetic acid as carbon source, the initial medium (1 l) contained 10 g l^{-1} yeast extract; 25 g l^{-1} sea salt, 8 g l^{-1} sodium acetate and a 10% (v/v) inoculum. Acetic acid (50 or 100%, w/w) was added via the pH-control system of the bioreactor in such a way that the pH of the cultures was maintained at 6.5 ± 0.1. The acetic acid used in the feed was not sterilised.

CO_2 and O_2 percentages of the ingoing and outgoing gas flows were determined with a Uras 10E gas analyser (Hartmann&Braun, Delft, The Netherlands). The gas flow was measured with an ADM 1000 Intelligent Flowmeter (J&W Scientific, Folsom, USA).

Analysis of biomass concentration and lipid analysis

Culture samples of 20-40 ml were centrifuged (17,000 *g*, 10 min). The cell pellet was washed in 25 ml demineralised water, lyophilised and weighed in order to determine the biomass concentration. Yield calculations were estimated after a correction for sampling. However,

accurate yield calculations would require the use of larger bioreactors, in which sampling has a smaller effect on culture volume. For lipid extraction, lyophilised biomass (100 mg) was incubated for 24 h in 5 ml of an extraction solution (chloroform:methanol=2:1, containing 0.5 mg butylated hydroxytoluene and, as an internal standard, 1 mg ml^{-1} methyl docosanoate) at room temperature with gentle stirring. The solution with the extracted lipids was separated from the cell debris by centrifugation. To the cell debris another 3 ml extraction solution was added and incubated a further 20 h. The solution with the extracted lipids was again separated from the cell debris by centrifugation and pooled with the solution of the first incubation. Methylated fatty acids were prepared from the lipid extract with trimethylsulphonium hydroxide (Butte 1983) and analysed by gas chromatography as previously described (de Swaaf et al. 1999). Values for the lipid content of the biomass and DHA content of the lipid are expressed in weight percentages.

Glucose measurements and viscosity measurements

Glucose in culture supernatants was determined using the glucose oxidase method (Trinder 1969; Boehringer, Mannheim, Germany). Culture viscosity was determined at 27 ^{o}C using a capillary Cannon-Fenske routine viscometer (Schott, Mainz, Germany) according to the manufacturers' instructions.

Results

Comparison of glucose- and acetic acid-grown fed-batch cultures

Production of biomass, total lipid and DHA were studied in glucose- and acetic acid-grown fed-batch cultures of *C. cohnii*. The composition of the complex medium was based on earlier studies on batch cultivation of this alga (de Swaaf et al. 1999).

At concentrations above 25 g l^{-1}, glucose inhibits growth of *C. cohnii* (de Swaaf et al. 1999) as well as lipid accumulation in shake-flask cultures (data not shown). To maintain the specific rate of glucose consumption during fed-batch cultivation close to its maximum value, the glucose feed was manually controlled to maintain a residual glucose concentration between 5 and 20 g l^{-1}. Over a period of 120 h, this cultivation yielded a final biomass concentration of 26 g l^{-1}

dry weight. During the first 24 h, growth was exponential with an estimated specific growth rate of 0.086 h^{-1}. After this period, the biomass concentration increased linearly with time, most probably as a result of an unidentified nutritional limitation (Fig. 1). During exponential growth, the lipid content of the biomass decreased from 8.2 to 3.7%. Then, as linear growth set in, the lipid content increased until after 90 h a maximum lipid content of 15% was reached. This lipid

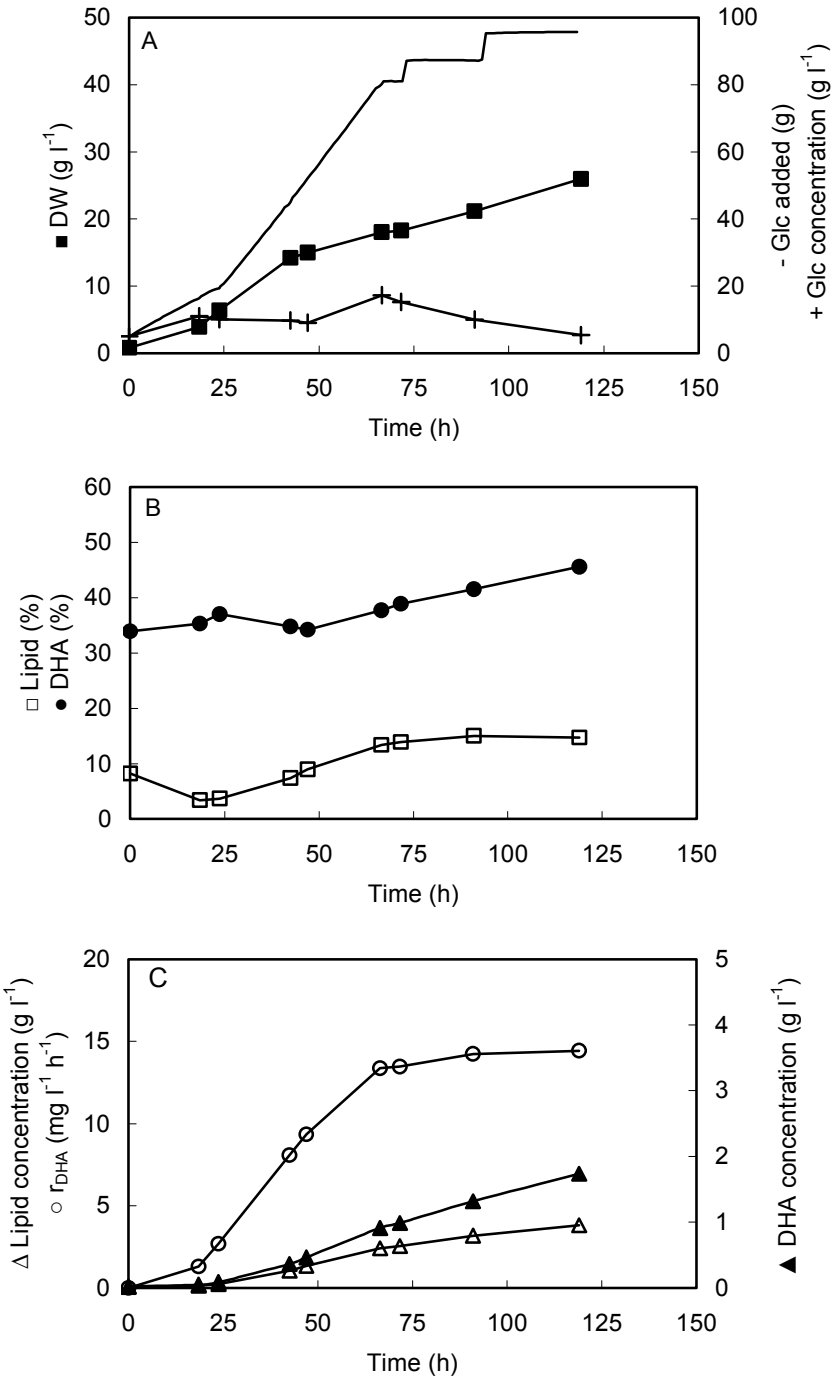

Fig. 1 Fed-batch cultivation of *C. cohnii* with a feed containing 50% (w/v) glucose. Initial medium: 10 g l^{-1} yeast extract, 25 g l^{-1} sea salt, 5 g l^{-1} glucose and 10% (v/v) inoculum.
A: Biomass dry weight (DW), glucose added (Glc added) and glucose concentration in the culture (Glc concentration).
B: Lipid content of dry biomass (Lipid) and DHA content of lipid (DHA).
C: Lipid concentration, overall volumetric productivity of DHA (r_{DHA}) and DHA concentration.

content was maintained until the end of the cultivation (Fig. 1). During the course of the cultivation, the DHA content of the lipids increased from 34 to 46%. The overall volumetric production rate of DHA (r_{DHA}), a key parameter for process optimisation, reached a maximum and constant value of 14 mg l^{-1} h^{-1} during the final 30 h of cultivation. The final lipid and DHA concentrations in the glucose-grown fed-batch cultures (Table 1) were 3.8 and 1.7 g l^{-1}, respectively.

Table 1 Comparison of acetic acid- and glucose-grown fed-batch cultures of *C. cohnii*. Selected parameters are shown for time point 120 h (glucose) and time point 210 h (acetic acid). Cultivation conditions are given in the Materials and Methods section. Fatty acids other than DHA are indicated as y:x where y represents the number of carbon atoms and x the number of double bonds.

	Feed			
	Glucose 50% (w/v)	Acetic acid 50% (w/w)	Acetic acid 100%	Acetic acid 100%, Glucanex
Time (h)	120	210	210	210
Biomass (g l^{-1})	26	51	61	56
Lipid content (% w/w)	15	54	49	51
Lipid concentration (g l^{-1})	3.8	28	30	29
12:0 in lipid (% w/w)	3	11	11	9
14:0 in lipid (% w/w)	18	26	25	25
16:0 in lipid (% w/w)	22	19	18	19
16:1 in lipid (% w/w)	-	2	2	2
18:0 in lipid (% w/w)	3	1	1	1
18:1 in lipid (% w/w)	8	11	11	11
DHA in lipid (% w/w)	46	29	32	32
DHA concentration (g l^{-1})	1.7	8.0	9.5	9.4
r_{DHA} (mg l^{-1} h^{-1})	14	38	45	44

To enable a comparison with the glucose fed-batch regime described above, the feed of an acetic acid-grown culture was also controlled to approach the maximum specific consumption rate on this substrate. This was done via the pH-stat technique, which controlled the supply of a

50% (w/w) acetic acid solution. During the first 48 h, growth was exponential, with a specific growth rate of 0.053 h^{-1}. As observed in the glucose-grown cultures, the increase of the biomass concentration became linear after this initial exponential growth phase (Fig. 2). The lipid content decreased during the first 24 h of cultivation on acetic acid. After this period, the lipid content

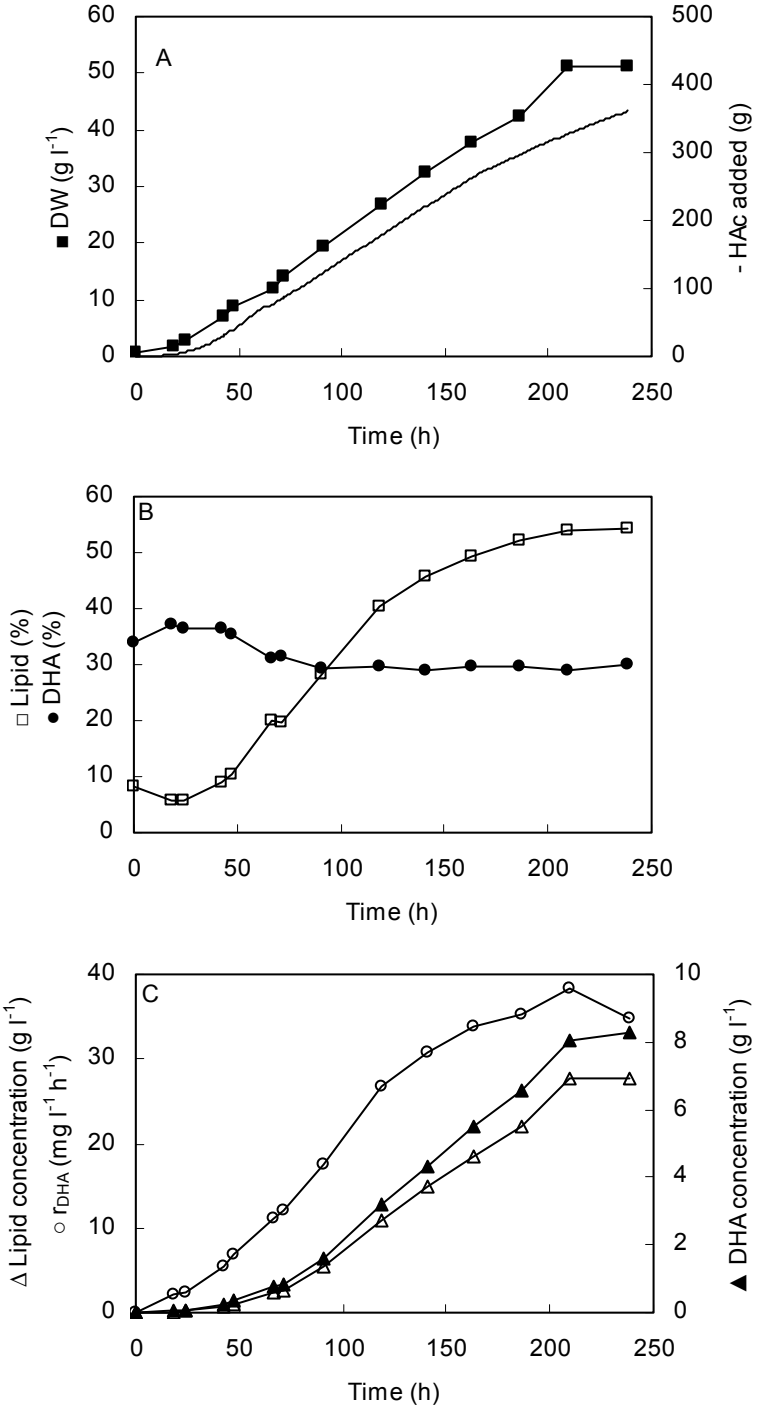

Fig. 2 Fed-batch cultivation of *C. cohnii* with a feed containing 50% (w/w) acetic acid. Initial medium: 10 g l^{-1} yeast extract, 25 g l^{-1} sea salt, 8 g l^{-1} NaAc and 10% (v/v) inoculum.
A: Biomass dry weight (DW) and acetic acid added (HAc added).
B: Lipid content of dry biomass (Lipid), DHA content of lipid (DHA).
C: Lipid concentration, overall volumetric productivity of DHA (r_{DHA}) and DHA concentration.

increased, especially between 48 and 150 h. The lipid content then leveled off, reaching a constant value of 54% between 210 and 240 h. The DHA content of the lipid varied between 29 and 38% during the initial phase of the fermentation, but remained constant at 30 ± 1% after 70 h (Fig. 2). The r_{DHA} was seen to reach a maximum of 38 mg l^{-1} h^{-1} at 210 h. At this stage of the fermentation, the concentrations of biomass, lipid and DHA were 51 g l^{-1}, 28 g l^{-1} and 8.0 g l^{-1}, respectively (Table 1). These biomass and lipid concentrations were higher than previously reported for *C. cohnii* (de Swaaf et al. 1999; Ratledge et al. 2001a). In view of the superior performance of the acetic acid-grown *C. cohnii* cultures relative to the glucose-grown cultures, it was attempted to further optimise the fed-batch fermentation process for cultivation on acetic acid.

Development of a high-cell-density fed-batch protocol

To further increase the final biomass and lipid concentrations, several modifications were introduced in the protocol for fed-batch cultivation of *C. cohnii* on acetic acid. To prevent unnecessary dilution, cultures were fed with pure acetic acid instead of a 50% (w/w) solution in water. Furthermore, the yeast extract was filter-sterilised to prevent heat inactivation of essential growth factors and a more efficient antifoaming agent (MAZU DF 8005 instead of silicone-SE2) was used.

The use of a pure acetic acid feed resulted in increased biomass concentrations during the first 210 h, relative to fed-batch cultivation with a 50% (w/w) acetic acid feed (Fig. 3, Table 1). This can be explained by the elimination of dilution, resulting from the presence of water in the feed. Otherwise, patterns of lipid accumulation and DHA content with a pure and 50% (w/w) acetic acid feeds differed by less than 10%. In the cultures grown with a pure acetic acid feed, prolonged feeding (>210 h) resulted in continued production of biomass. During this extended feed phase, the biomass lipid content and the DHA content of the lipid remained essentially constant (Fig. 3). The volumetric productivity of DHA in these cultures remained at a high and constant value of 46 ± 2 mg l^{-1} h^{-1} between 150 and 400 h. The estimated overall biomass yield on acetic acid, corrected for sampling, was 0.13 g dry biomass g^{-1} acetic acid. After 400 h cultivation, the concentrations of dry biomass, lipid and DHA were 109 g l^{-1}, 61 g l^{-1} and 19 g l^{-1}, respectively.

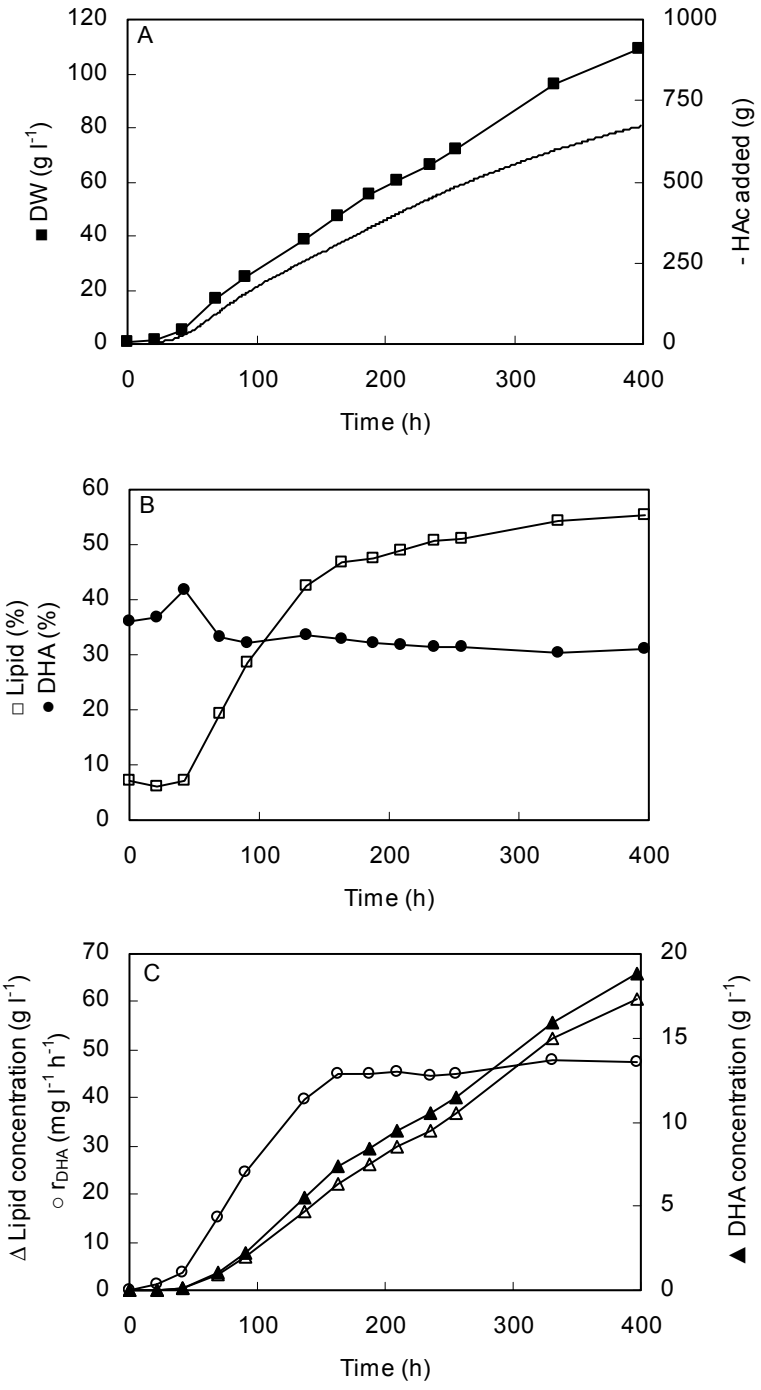

Fig. 3 Fed-batch cultivation of *C. cohnii* with a feed consisting of pure acetic acid. Initial medium: 10 g l^{-1} yeast extract, 25 g l^{-1} sea salt, 8 g l^{-1} NaAc and 10% (v/v) inoculum.

A: Biomass dry weight (DW) and acetic acid added (HAc added).

B: Lipid content of dry biomass (Lipid), DHA content of lipid (DHA).

C: Lipid concentration, overall volumetric productivity of DHA (r_{DHA}) and DHA concentration.

During this high-cell-density fermentation process, the dissolved oxygen tension could only just be maintained above 30% of air saturation as the stirrer speed had to be increased to 1250 rpm in 200 h (Fig. 4) and remained above 1100 rpm for most of the cultivation time. The need for vigorous stirring (at a fixed aeration rate of 1 vvm) was at least partly due to the production of a

viscous extracellular polysaccharide by *C. cohnii* (de Swaaf et al. 2001). This presents a problem for scaling up of the process, as efficient gas transfer is more difficult to achieve in large-scale cultivations than in small-scale laboratory fermenters (Einsele 1978; Oosterhuis and Kossen 1983).

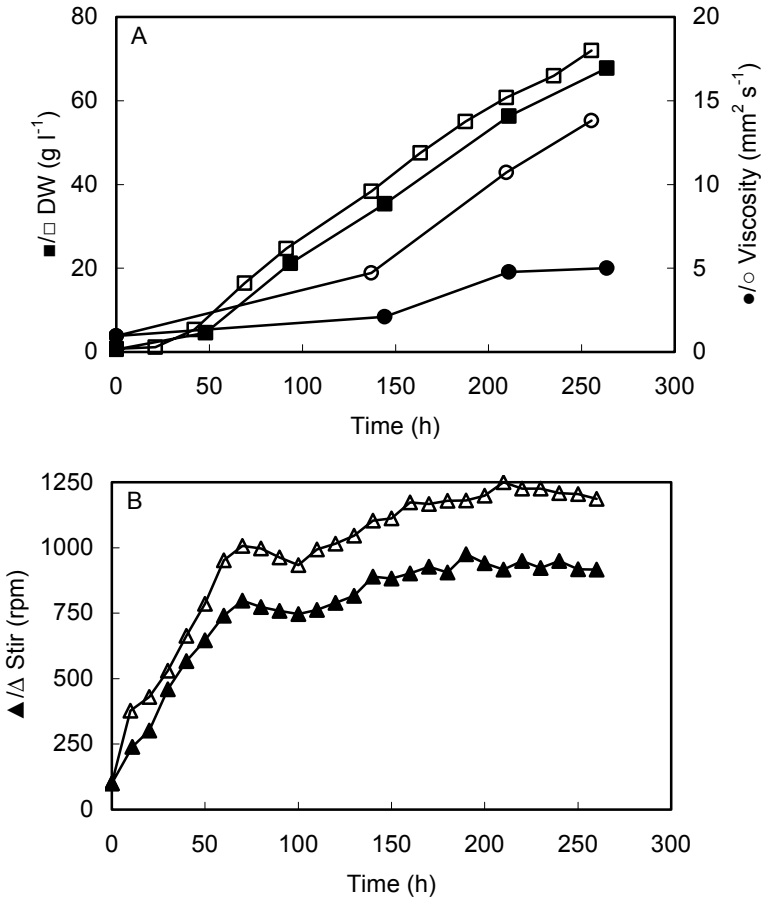

Fig. 4 Effect of the addition of the polysaccharide-hydrolysing enzyme preparation Glucanex® on fed-batch cultivation of *C. cohnii*. Cultures were fed with pure acetic acid. Initial medium: 10 g l^{-1} yeast extract, 25 g l^{-1} sea salt, 8 g l^{-1} NaAc and 10% (v/v) inoculum. Cultures were grown either in the presence (closed symbols) or absence (open symbols) of 0.5 g l^{-1} Glucanex.

A: Biomass dry weight (DW) and viscosity.

B: Stirrer speed (Stir).

Reduction of viscosity in high-cell-density cultures

In an attempt to decrease culture viscosity in the high-cell-density fed-batch cultures of *C. cohnii*, the commercial polysaccharide-hydrolysing enzyme preparation Glucanex® (Novo Nordisk, Neumatt, Switzerland) was added to cultures. In a preliminary experiment, addition of Glucanex (1.0 g l^{-1}) to a fed-batch culture led to an immediate increase of the dissolved oxygen tension and, consequently, an automatic reduction of the stirrer speed. In this preliminary experiment,

Glucanex addition did not influence the rates of acetic acid consumption and respiration, suggesting that overall metabolic activity was unaffected (data not shown).

In a cultivation, in which 0.5 g l^{-1} Glucanex was added at the start of the cultivation, the viscosity of the culture supernatant was strongly reduced as compared to a cultivation without Glucanex (Fig. 4). The stirrer speed remained below 1000 rpm and was even below 900 rpm during most of the process. Doubling of the Glucanex concentration did not result in a further decrease of the stirring rate. Patterns of O_2 consumption, CO_2 production, acetic acid addition (not shown), biomass concentration (Fig. 4), lipid accumulation and fatty acid profiles (Table 1) were similar to those observed in cultures growth without addition of Glucanex.

Discussion

High-cell-density fed-batch cultivation of *C. cohnii*

Examples of high-cell-density cultivation, reaching over 100 g dry biomass per litre of cultivation broth, have been reported previously for bacteria, archaea, yeasts and filamentous fungi (Riesenberg and Guthke 1999) but not, to our knowledge, for microalgae. Fed-batch cultivation of *C. cohnii* on a medium with yeast extract, sea salt and sodium acetate and a feed of pure acetic acid, resulted in a final biomass concentration of 109 g l^{-1} dry weight. Even at these high biomass densities, DHA production continued to occur. This, for the first time, demonstrates that it is possible to grow this heterotrophic alga in high-cell-density cultures. High contents of total lipid and DHA were retained in the high-cell-density cultures, leading to maximum product concentrations of 61 g l^{-1} (total lipid) and 19 g l^{-1} (DHA). This is likely to result in more cost effective product recovery than was possible in previously reported low-cell-density batch cultures (de Swaaf et al. 1999).

In this first study on high-cell-density cultivation of *C. cohnii*, several aspects appeared essential to reach high-cell-density. Firstly, a cultivation period of 400 h was required. Furthermore, the proper selection of the carbon source was crucial, namely acetic acid. Finally, the high intracellular accumulation of storage materials (such as lipids) contributed to the dry biomass and thus facilitated high-cell-density cultivation. Not only the carbon source but also the medium composition is important for the accumulation process(es) in *C. cohnii* (de Swaaf et al.

1999; de Swaaf et al. 2001). In general, lipid accumulation in microorganisms is stimulated by an excess of a carbon source and a limitation in one (or more) of the other nutrients, especially nitrogen. Microbial lipid accumulation is often a biphasic process. In the first phase exponential cell division occurs and in the second phase the growth rate decreases (due to a nutritional limitation) and lipids start to accumulate (Leman 1997).

This study did not aim at a quantitative stoichiometric analysis of substrate consumption and the formation of biomass and lipids. For such quantitative studies, several modifications to the experimental set-up are required. Firstly, larger fermenters should be used to minimise the impact of sampling on analytical procedures (especially gas analysis). Secondly, water balancing would need to be addressed. For example, in the high-cell-density fermentation shown in Fig. 3, an estimated 0.4 l water was produced from the 670 g of pure acetic acid fed to the culture. In the prolonged fermentation experiments, this increase in culture volume was compensated for by two factors. Firstly, sampling removed a significant fraction of the culture broth during fermentation. Secondly, a significant amount of water was lost by evaporation as a result of aeration with dry air. Even a cooled condenser cannot prevent loss of water via the exhaust gas. Assuming that the exhaust gas was water-saturated and cooled to the temperature of the condenser, ca. 0.3 l water was lost during 400 h of cultivation. Quantification of this water loss is essential for future work on the quantitative analysis of product-formation stoichiometries in high-cell-density cultures of *C. cohnii*.

High-cell-density cultivation on acetic acid resulted in a high oxygen demand. To maintain aerobic conditions, a very high stirrer speed had to be maintained during a large part of the process. Oxygen transfer is likely to be a limiting factor during a commercial-scale high-cell-density cultivation of *C. cohnii*. Furthermore, the less efficient heat transfer in large-scale reactors may result in cooling problems due to the combination of biological heat production and power input via mixing. The latter factor will be enhanced by the production of extracellular polysaccharides by *C. cohnii*, which strongly increase culture viscosity (de Swaaf et al. 2001). Our results (Fig. 4) demonstrate that, until polysaccharide-negative strains of *C. cohnii* have been isolated, viscosity can be reduced by addition of a food-grade polysaccharide hydrolase preparation to the fed-batch cultures. This facilitated oxygen transfer, as evident from the reduced stirrer speeds required to sustain a sufficient dissolved oxygen tension. Moreover, due to the reduced viscosity, less power input is required and consequently, less heat is generated.

Use of acetic acid as a carbon source for production of DHA in high-cell-density fed-batch cultures of *C. cohnii* resulted in much higher lipid and DHA contents than in cultivations on glucose (de Swaaf et al. 1999; Jiang and Chen 2000). This difference may be related to the biochemistry and subcellular location of acetyl-CoA metabolism, a subject about which very little is known for *C. cohnii*. It is likely that, similar to the situation in yeasts (Pronk et al. 1996), the mitochondrial pyruvate-dehydrogenase complex is the main source of acetyl-CoA during growth on glucose. The fatty acid synthetase complex from *C. cohnii* was shown to be cytosolic (Sonnenborn and Kunau 1982) which suggests that, similar to the situation in yeasts (Ratledge and Evans 1989), lipid synthesis in this alga occurs in the cytosol. This implies that, during growth on glucose, export of acetyl-CoA from the mitochondrial matrix to the cytosol is required to make it available for lipid synthesis. In contrast acetate can be directly activated to acetyl-CoA by the action of acetyl-Coenzyme A synthetase. In yeasts at least one of the isoenzymes of acetyl-CoA synthetase occurs in the cytosol (de Jong-Gubbels 1998). If this is also the case in *C. cohnii*, this would undo the need for translocation of acetyl-CoA from the mitochondrial matrix. Studies on the biochemistry, subcellular compartmentation and regulation of acetyl-CoA metabolism in *C. cohnii* are a prerequisite for understanding DHA production by this alga.

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References

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6:123-129

Berg MA van den, Jong-Gubbels P de, Kortland CJ, Dijken JP van, Pronk JT, Steensma HY (1996) The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. J Biol Chem 271:28953-28959

Butte W (1983) Rapid method for the determination of fatty acid profiles from rats and oils using trimethylsulphonium hydroxide for transesterification. J Chromatogr 261:142-145

Einsele A (1978) Scaling up bioreactors. Proc Biochem 13 (7):13-14

Harrington GW, Holz GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochem Biophys Acta 164:137-139

Hornstra G (2000) Essential fatty acids in mothers and their neonates. Am J Clin Nutr 71:1262-1269

Jiang Y, Chen F (2000) Effects of medium glucose concentration and pH on docosahexaenoic acid content of heterotrophic *Crypthecodinium cohnii*. Process Biochem 35:1205-1209

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. Lipid Technol 8:107-110

Leman J (1997) Oleaginous microorganisms: an assessment of the potential. Adv Appl Microbiol 43:195-243

Martinez-Blanco H, Reglero A, Fernandez-Valverde M, Ferrero MA, Moreno MA, Penalva MA, Luengo JM. (1992) Isolation and characterization of the acetyl-CoA synthetase from *Penicillium chrysogenum*. Involvement of this enzyme in the biosynthesis of penicillins. J Biol Chem 267:5474-5481

Nettleton JA (1993) Are n-3 fatty acids essential nutrients for fetal and infant development? J Am Dietetic Assoc 93:58-64

Oosterhuis NMG, Kossen NWF (1983) Oxygen transfer in a production scale bioreactor. Chem Eng Res Des 61:308-312

Preez JC du, Immelman M, Kock JLF, Killian SG (1995) Production of gamma-linolenic acid by *Mucor circinelloides* and *Mucor rouxii* with acetic acid as carbon substrate. Biotechnol Lett 17:933-938

Pronk JT, Steensma HY, Dijken JP van (1996) Pyruvate metabolisme in *Saccharomyces cerevisiae*. Yeast 12:1607-1633

Ratledge C, Evans CT (1989) Lipids and their metabolism. In: Rose AH, Harrison JS (eds) The Yeasts, Vol. 3, pp. 367-455. Academic Press, New York

Ratledge C, Kanagachandran K, Anderson AJ, Grantham DJ, Stephenson JC (2001a) Production of docosahexaenoic acid (DHA) by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. Lipids 36:1241-1246

Ratledge C, Anderson AJ, Kanagachandran K, Grantham DJ, Stephenson JC, Swaaf ME de, Sijtsma L (2001b) Culture of *Crypthecodinium cohnii* for the synthesis of a polyunsaturated fatty acid. PCT International Application 2001 (Jan 18) WO 01/04338 A1; PCT/GB00/0295

Riesenberg D, Guthke R (1999) High-cell-density cultivation of microorganisms. Appl Microbiol Biotechnol 51:422-430

Sijtsma L, Springer J, Meesters PAEP, Swaaf ME de, Eggink G (1998) Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae. Recent Res Dev Microbiol 2:219-232

Swaaf ME de, Rijk TC de, Eggink G, Sijtsma L (1999) Optimisation of docosahexaenoic acid production in batch cultivations by *Crypthecodinium cohnii*. J Biotechnol 70:185-192

Swaaf ME de, Grobben GJ, Eggink G, Rijk TC de, Meer P van der, Sijtsma L (2001) Characterisation of extracellular polysaccharides produced by *Crypthecodinium cohnii*. Appl Microbiol Biotechnol 57:395-400

Sonnenborn U, Kunau WH (1982) Purification and properties of the fatty acid synthetase complex from the marine dinoflagellate, *Crypthecodinium cohnii*. Biochim Biophys Acta 712:523-534

Trinder P (1969) Determination of glucose in blood using glucose oxidase with alternative oxygen acceptor. Ann Clin Biochem 6:24

Woodward J, Merrett MJ (1975) Induction potential for glyoxylate cycle enzymes during the cell cycle of *Euglena gracilis*. Eur J Biochem 15:555-559

Chapter 5

Production of docosahexaenoic acid rich biomass by fed-batch cultivation of *Crypthecodinium cohnii* in a 150-l bioreactor on acetic acid

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The work described in this chapter was performed at Norferm DA.

Abstract

In laboratory-scale experiments, docosahexaenoic acid (DHA), a polyunsaturated fatty acid with applications in foods and pharmaceuticals, can be produced at a high overall volumetric rate by cultivation of *Crypthecodinium cohnii* on acetic acid. For commercial implementation, scaling up is required. In this chapter, fed-batch cultivation of *C. cohnii* on acetic acid on pilot-plant scale (a 150-l bioreactor with 100-l working volume) and processing of the biomass are investigated. A procedure was developed to produce sufficient and viable cells to inoculate the 150-l bioreactor. *C. cohnii* was cultivated for 188 h in the 150-l bioreactor, yielding a final biomass concentration of 38 g dry weight l^{-1} with a DHA content of 12%. Centrifugation resulted in a 2.2 fold concentration of the dry matter in the heavy fraction. Subsequent spray-drying of the heavy fraction did not significantly affect the DHA content of the lipid.

Introduction

Docosahexaenoic acid (DHA) is a ω-3 polyunsaturated fatty acid with applications in foods and pharmaceuticals (Barclay et al. 1994). As the normal growth and development of several marine fish larvae depends on the presence ω-3 PUFAs in their diet, particularly DHA and eicosapentaenoic acid (Rodríguez et al. 1998), DHA can also be applied for large-scale marine fish farming.

The heterotrophic marine microalga *Crypthecodinium cohnii* can accumulate lipids with a high fraction (30-50%) of DHA, with other PUFAs accounting for less than 1% of the total lipid content (Harrington and Holz 1968). Important factors for the economic feasibility of fermentative DHA production with *C. cohnii* are the cultivation scale and the volumetric productivity (r_{DHA}; Sijtsma et al. 1998). Biomass concentration, lipid content of the cells, DHA content of the lipid and cultivation time are the factors that determine r_{DHA}.

Current DHA production processes with *C. cohnii* use glucose as carbon source (Kyle et al. 1996). In batch cultivations, the maximum r_{DHA} reported on glucose is 19 mg l^{-1} h^{-1} (de Swaaf et al. 1999). Similar productivities were observed in fed-batch cultivations grown with a concentrated (50% w/v) glucose feed (de Swaaf et al. 2003). In pH-controlled fed-batch cultivations with 50% (w/w) acetic acid as the carbon source, productivities of up to 38 mg l^{-1} h^{-1} were achieved on lab-scale (Ratledge et al. 2001; de Swaaf et al. 2003). The r_{DHA} could be further raised on lab-scale to 48 mg l^{-1} h^{-1} with pure acetic acid as feed and prolonged cultivation (de Swaaf et al. 2003). Therefore, acetic acid may be an industrially relevant carbon source for the production of DHA by *C. cohnii*. However, as the productivities reported above were obtained in 2-l laboratory fermentations, scale-up experiments are required before commercial implementation can be contemplated.

The main goal of this study was to scale up the protocol for fed-batch cultivation of *C. cohnii* on acetic acid from 2-l to a 150-l scale, as an important first step towards the development of an industrial large-scale (>50 m^3) cultivation protocol. Furthermore, centrifugation and spray-drying were evaluated as methods for harvesting and drying the biomass, respectively.

Materials and Methods

Strain and maintenance

Crypthecodinium cohnii ATCC 30772 was maintained by sub-cultivation on a complex medium containing 2 g l^{-1} yeast extract (Quest International, Naarden, The Netherlands); 9 g l^{-1} glucose and 25 g l^{-1} sea salt (Sigma-Aldrich, Zwijndrecht, The Netherlands). Cultures were incubated statically at 25 ^{o}C. The inoculum size in all cultivations was 10% (v/v). All medium components were heat-sterilised separately (121 ^{o}C, 20 min) unless indicated otherwise.

Preparation of inocula for pilot-scale fermentations

In order to produce a sufficient amount of viable biomass to inoculate the 150-l bioreactor, the following procedure was applied.

Step 1. Static cultures used for strain maintenance (see above) were grown for 4-10 days and used to inoculate shake-flask cultures. For shake-flask cultivation, 50 ml cultures (2 g l^{-1} yeast extract, 25 g l^{-1} sea salt, 9 g l^{-1} glucose and 5 ml inoculum) were incubated for 3 days in 300 ml erlenmeyer flasks in an orbital shaker (Gallenkamp cooled orbital shaker, 150 rpm, 27 ^{o}C).

Step 2. Shake-flask cultures were incubated for 3 days in 500 ml medium (5.5 g l^{-1} yeast extract, 25 g l^{-1} sea salt, 25 g l^{-1} glucose and 50 ml inoculum from step 1) in 3-l baffled erlenmeyer flasks in an orbital shaker.

Step 3. In 15-l bioreactors fed-batch cultures with a start volume of 8 l (6.5 g l^{-1} yeast extract, 22 g l^{-1} sea salt, 6.9 g l^{-1} sodium acetate and 500 ml inoculum from step 2) were incubated until an OD470 of 10±2 was reached. Details are described below.

Step 4. The final pilot-plant cultivation in a 150-l bioreactor with a start volume of 78 l (9.0 g l^{-1} yeast extract, 22.4 g l^{-1} sea salt, 7.2 g l^{-1} sodium acetate and 8 l inoculum from step 3) was incubated for 188 h. Further details are described below.

Cultivations in 15-l and 150-l bioreactors

Fed-batch cultivation was performed at 27 ^{o}C in 15-l and 150-l bioreactors (Chemap AG, Volketswil, Switserland). The dissolved oxygen tension (DOT) was kept above 30% of air saturation by manually adjusting the stirrer speed and by manually controlling the (filter-sterilised) air flow into the bioreactor. Foam production in the cultivations was repressed by addition of 1-2 droplets antifoam (MAZU DF 8005, BASF, Cheadle, UK) per litre of initial culture volume. The pH was controlled at 6.5 by automatic addition of filter-sterilised (Sartopore 2 membrane filters, Sartorius AG, Goettingen, Germany) 60% (w/w) acetic acid (HAc). Samples (10-30 ml) were taken every two days for biomass analysis. After 96 h and 188 h of cultivation in the 150-l bioreactor, 20 l and the total final 97 l of cultivation broth, respectively, were frozen at –29 ^{o}C and used for biomass-processing experiments.

Measurement of biomass concentration, lipid analysis and determination of the content of dry matter, ash, protein and sugar

Measurement of biomass concentration and lipid analysis were performed as previously described (de Swaaf et al. 2003). Dry matter (including dissolved compounds such as salts) was determined after overnight drying of culture samples at 105 °C.

The ash content was calculated from sample weights before and after drying. Samples were heated at 550 ^{o}C for at least 3 h and cooled to room temperature. A few droplets of an ammonium nitrate solution (20% w/v) were added and samples were heated at 105 ^{o}C for 1 h. Subsequently, samples were heated for another 3 h at 550 ^{o}C.

Protein content was determined by an elemental analyser according to the instructions from the manufacturer (Fisons instruments, Beverly, MA, USA). In this analyser, carbon, hydrogen, nitrogen and organic linked sulphur are detected as N_2, CO_2, H_2O and SO_2, respectively, by gas chromatography after combustion at 1000°C and passage over a column containing an oxidative catalyst consisting of copper oxide and copper. The protein content was calculated by multiplying the N-content by the Kjeldahl-factor (6.25).

Sugar content of samples was determined by a colorimetric method (Anthrone method; Herbert et al. 1971).

Centrifugation and spray-drying

The frozen culture was thawed overnight at 2-4 ^{o}C in a waterbath. The thawed cultivation broth was then centrifuged at 9800 rpm by a Westfalia separator (SA type 1-02-175, Westfalia, Oelde, Germany) at an overall average flow of 60 l h^{-1}.

Both the light and the heavy fractions were dried in a spray-drier (APV systems PSD52, Søborg, Denmark). The spray-drier was equipped with a centrifugal nozzle and was operated at an inlet temperature of 180 ^{o}C and an outlet temperature of 90 ^{o}C. The centrifugal nozzle had a speed of 25,000 rpm. The temperature of feed at the inlet was 20-23 ^{o}C. The spray-dried materials were stored at –20 ^{o}C prior to chemical analysis.

Results and discussion

Cultivation in a 150-l bioreactor

The aim of the experiments was to scale-up a previously reported 2-l lab-scale fed-batch cultivation of *C. cohnii* on 50% (w/w) acetic acid (de Swaaf et al. 2003) to a 150-l bioreactor. In the 150-l bioreactor, the initial concentrations of yeast extract, sea salt and sodium acetate were 11% lower than in the 2-l experiments and a slightly higher acetic acid content in the feed of 60% (w/w) was used (de Swaaf et al. 2003).

The specific growth rate of the cultivation in a 150-l bioreactor reached a maximum of 0.048 h^{-1} during the first 40 h of cultivation. This value is close to the maximum specific growth rate (0.053 h^{-1}) observed in the 2-l scale cultivations, indicating that the inoculum contained a sufficient number of viable cells. After 40 h, the specific growth rate decreased due to a nutritional limitation (Fig. 1A). To prevent overflow of the bioreactor, 20 l was taken from the bioreactor after 93 h. The cultivation was terminated after 188 h. At this time point, 26.7 kg acetic acid was added and the dry biomass concentration was 38 g l^{-1}. This is close to the biomass concentration of 42 g l^{-1} at 186 h observed in the 2-l scale fermentations (Fig. 1A).

The lipid content of the cells decreased during the first 40 h and, subsequently, increased until after 136 h the lipid content was 49% (Fig. 1B). The DHA content of the lipid varied between 23 and 30% but remained constant at 29±1% after 40 h (Fig. 1C). At approximately 140

h, the values for dry weight, r_{DHA}, lipid and DHA concentrations of the 150-l and 2-l scale cultivations differed by less than 10% (Table 1). The exact lipid content of the cells at the end of the cultivation on pilot-plant scale is unknown as the end sample taken for lipid analysis was lost. Nevertheless, the lipid content of the cells, DHA content of the lipid and DHA content of the cells at 188 h were at least 45%, 27% and 12% respectively (as calculated from Table 2).

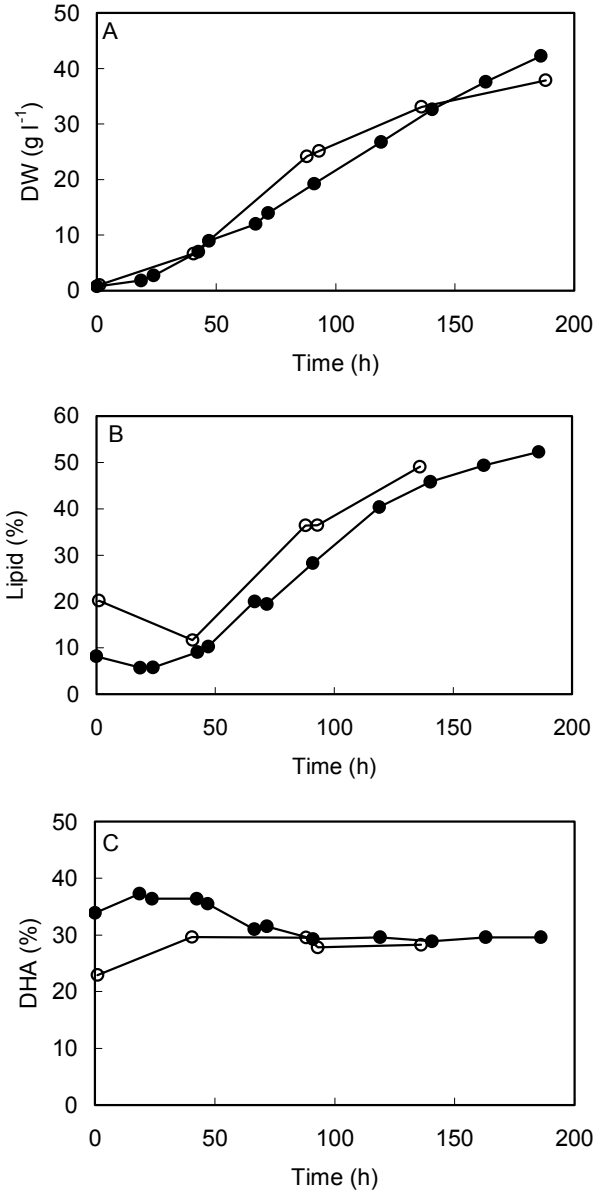

Fig. 1 Comparison of fed-batch cultures of *C. cohnii* in 2-l (●) and 150-l (○) bioreactors grown on 50 and 60% (w/w) acetic acid, respectively. Data from 2-l scale acetic acid-grown fed-batch cultures of *C. cohnii* were taken from Chapter 4. Cultivation conditions are given in the Materials and Methods section.
A: Biomass dry weight (DW).
B: Lipid content of dry biomass (Lipid).
C: DHA content of the lipid.

Table 1 Comparison of fed-batch cultures of *C. cohnii* in 2-l and 150-l bioreactors grown on 50 and 60% (w/w) acetic acid, respectively. Selected parameters are shown for time points 140 h (2-l bioreactor) and 136 h (150-l bioreactor). Data from 2-l scale acetic acid-grown fed-batch cultures of *C. cohnii* were taken from Chapter 4. Cultivation conditions are given in the Materials and Methods section. Fatty acids other than DHA are indicated as y:x where y represents the number of carbon atoms and x the number of double bonds.

	Bioreactor	
	2-l	150-l
Time (h)	140	136
Biomass (g l^{-1})	33	33
Lipid content (% w/w)	46	49
Lipid concentration (g l^{-1})	15	16
12:0 in lipid (% w/w)	11	9
14:0 in lipid (% w/w)	26	26
16:0 in lipid (%w/w)	20	20
16:1 in lipid (%w/w)	2	2
18:0 in lipid (%w/w)	1	2
18:1 in lipid (%w/w)	11	13
DHA in lipid (%w/w)	29	28
DHA concentration (g l^{-1})	4.3	4.6
r_{DHA} (mg l^{-1} h^{-1})	31	34

Centrifugation and spray-drying

Centrifugation and spray-drying were evaluated as methods to harvest and dry the algal cells.

By centrifugation of the 97 l cultivation broth the dry matter was concentrated by a factor of 2.2. The dry matter content increased from 6.7% in the cultivation broth to 14.7% (w/w) in the heavy fraction. Concentration of the biomass proceeded in a straightforward manner and did not lead to any notable problems (Lars Mejdal, Norferm DA, personal communication).

Both the light and the heavy fraction were spray-dried after centrifugation. Spray-drying of the heavy fraction resulted in four dried materials. Part of the heavy fraction had gone directly through the spray-dryer (heavy fraction I). To collect most of the dried cells, materials were also collected inside the spray-dryer after spray-drying. Material was taken from the lower part of the

spray-dryer (heavy fraction II), the cyclone and the powder channel (heavy fraction III) and from the upper part of the spray-dryer (heavy fraction IV). Materials were analysed separately to determine whether they had similar DHA contents.

Table 2 The overall composition of spray-dried materials. Part of the concentrate had gone directly through the spray-dryer (heavy fraction I). Material on the lower part of the spray-dryer was taken after spray-drying (heavy fraction II). Material from the cyclone and the powder channel was taken out after spray-drying and pooled (heavy fraction III). Finally, material was collected after spray-drying, from the upper part of the spray-dryer (heavy fraction IV). Spray-dried light fraction was collected (light fraction). Data are represented in percentages of total dry matter except for the DHA content of the lipid (percentages of total lipid) and the total weight values in kg.

	Heavy fraction I	Heavy fraction II	Heavy fraction III	Heavy fraction IV	Light fraction
Dry matter	100	99	99	98	95
Ash	10	9	11	9	49
Lipid	47	43	44	43	6
DHA of the lipid	28	25	28	19	25
Crude protein	5	4	5	4	7
Total sugar	33	34	35	29	20
Total weight (kg)	1.4	1.0	0.6	0.2	0.5

After the spray-drying procedure, the water content of all dried fractions was less than 5%. The overall composition of the dried materials, including the dried light fraction, was determined. In Table 2, dry matter, ash, lipid, DHA of the lipid, crude protein and total sugar percentages are given of the various dried materials. The lipid was found mostly in the heavy fraction after centrifugation and spray-drying whereas a very small amount of lipid was present in the dried light fraction. The DHA content of the lipid in the dried heavy fraction materials I, II and III varied between 25 and 28%. The DHA content of the lipid in dried heavy fraction IV was significantly lower namely 19%. This may be due to oxidation, as the material was collected from the area in the spray-dryer with the highest temperatures.

Conclusions

The results obtained in fed-batch cultivations of *C. cohnii* on acetic acid at pilot-plant and lab-scale were highly similar. This represents an important step forward towards commercial-scale production of DHA-rich biomass via this cultivation mode. Centrifugation and spray-drying at pilot-plant scale of the produced algal material proceeded without notable problems. Spray-drying appeared a suitable method to dry the algal material. The dried material fractions I, II and III were pooled (totally 3.2 kg) and used for initial tests in feed formulations for fish larvae. These studies are beyond the scope of this thesis.

Acknowledgements

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References

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6:123-129

Harrington GW, Holz GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochim Biophys Acta 164:137-139

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. In: Norris JR, Ribbons DW (eds) Methods of microbiology 5b. Academic Press, New York, pp 267-269

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. Lipid Technol 8:107-110

Ratledge C, Kanagachandran K, Anderson AJ, Grantham DJ, Stephenson JC (2001) Production of docosahexaenoic acid (DHA) by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. Lipids 36:1241-1246

Rodríguez C, Pérez JA, Badía P, Izquierdo MS, Fernández-Palacios H, Hernández L (1998) The n-3 highly unsaturated fatty acids requirements of gilthead seabram (*Sparus aurata* L.)

larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture 169:9-23

Sijtsma L, Springer J, Meesters PAEP, Swaaf ME de, Eggink G (1998) Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae. Recent Res Dev Microbiol 2:219-232

Swaaf ME de, Rijk TC de, Eggink G, Sijtsma L (1999) Optimisation of docosahexaenoic acid production in batch cultivations by *Crypthecodinium cohnii*. J Biotechnol 70:185-192

Swaaf ME de, Sijtsma L, Pronk JT (2003) High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*. Biotechnol Bioeng 81:666-672

Chapter 6

Fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii* on ethanol

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The heterotrophic marine microalga *Crypthecodinium cohnii* produces docosahexaenoic acid (DHA), a polyunsaturated fatty acid with food and pharmaceutical applications, when grown on complex media with yeast extract, sea salt and a suitable carbon source. So far, DHA production has been studied with glucose and acetic acid as carbon sources. This study investigates the potential of ethanol as an alternative carbon source for DHA production by *C. cohnii*. In shake-flask cultures, the alga was able to grow on media consisting of yeast extract, sea salt and ethanol. The specific growth rate was optimal with 5 g l^{-1} ethanol and did not occur at 0 g l^{-1} and above 15 g l^{-1}. In fed-batch cultivations with a controlled feed of pure ethanol, the cumulative ethanol addition could be much higher than 15 g l^{-1}, thus enabling a high final cell density and DHA production. The dissolved oxygen tension (DOT) was used to assess whether ethanol was limiting or inhibitory. The feed rate was increased when the DOT decreased after a small manual pulse of ethanol and was temporarily stopped when the DOT rose due to inhibition by high ethanol concentrations. In a representative fed-batch cultivation of *C. cohnii* with pure ethanol as the feed, 83 g l^{-1} dry biomass, 35 g l^{-1} total lipid and 12 g l^{-1} DHA were produced in 220 h. The overall volumetric productivity of DHA was 53 mg l^{-1} h^{-1}, which is the highest value so far reported for this alga.

Introduction

The ω-3 long-chain polyunsaturated fatty acids (PUFAs) are valuable ingredients of food and pharmaceutical products. As PUFAs stimulate visual and neurological development in preterm and young children (Nettleton 1993; Hornstra 2000), they are included in various infant foods (Barclay et al. 1994). Fish oil, the traditional source of PUFAs, is a limited resource of variable composition and quality. Therefore, microbial processes for production of PUFAs, and in particular docosahexaenoic acid (DHA, 22:6), are of considerable economic interest (Sijtsma et al. 1998).

Under appropriate cultivation conditions, the heterotrophic marine microalga *Crypthecodinium cohnii* can accumulate lipids with a high fraction (30-50%) of DHA, with other PUFAs accounting for less than 1% of the total lipid content (Harrington and Holz 1968). Cultivation scale and volumetric productivity (r_{DHA}) have been identified as major factors in determining the economic feasibility of fermentative DHA production with *C. cohnii* (Sijtsma et al. 1998). Factors that determine r_{DHA} are biomass concentration, lipid content of the cells, DHA content of the lipid and cultivation time. Obviously, a high DHA content of the biomass is also desirable from the viewpoint of product recovery.

Current DHA production processes with *C. cohnii* use glucose as carbon source (Kyle et al. 1996). In batch cultivations, the maximum r_{DHA} reported on glucose is 19 mg l^{-1} h^{-1} (de Swaaf et al. 1999). Similar productivities were observed in fed-batch cultivations grown with a concentrated (50% w/v) glucose feed (de Swaaf et al. 2003). In pH-controlled fed-batch cultivations with acetic acid as the carbon source, productivities of up to 48 mg l^{-1} h^{-1} were achieved (Ratledge et al. 2001; de Swaaf et al. 2003). This clearly indicates that the carbon source can have a strong impact on DHA productivity by *C. cohnii*.

The present paper investigates the use of ethanol as a carbon source. Like acetic acid, metabolism of ethanol in eukaryotes proceeds via acetyl-coenzyme A (Woodward and Merrett 1975; Martinez-Blanco et al. 1992; van den Berg et al. 1996). As a carbon source for large-scale cultivation, ethanol may be more attractive than acetic acid due to its lower cost (Tilton 2002) and the fact that it is less corrosive than acetic acid (Lide 1997). Moreover, the biomass yield of microorganisms, both per mole of carbon substrate and per mole of oxygen consumed, is generally higher during growth on ethanol than during growth on acetic acid (Verduyn et al.

1991; Linton and Rye 1989). Although an early study on *C. cohnii* reports that this alga is capable of growth on ethanol (Provasoli and Gold 1962), quantitative data on growth and DHA production by *C. cohnii* during growth on ethanol are currently not available.

The aim of this study was to investigate whether ethanol is of interest as a carbon source for the industrial production of DHA with *C. cohnii.* To this end, the optimum concentration range for growth on ethanol was investigated in batch cultures. Subsequently, fed-batch cultivation was used to evaluate growth and DHA production under industrially relevant conditions.

Materials and methods

Strain and maintenance

Crypthecodinium cohnii ATCC 30772 was maintained by subcultivation on a complex medium containing 2 g l^{-1} yeast extract (Oxoid, Basingstoke, UK); 9 g l^{-1} glucose and 25 g l^{-1} sea salt (Sigma-Aldrich, Zwijndrecht, The Netherlands). Cultures were incubated statically at 25 ^{o}C. The inoculum size in all cultivations was 10% (v/v). All medium components were heat-sterilised separately (121 ^{o}C, 20 min) unless indicated otherwise.

Shake-flask cultivation

Static cultures were grown for 4-10 d and then used to inoculate 50 ml medium (2 g l^{-1} yeast extract; 25 g l^{-1} sea salt and 9 g l^{-1} glucose) in 300-ml erlenmeyer flasks. These flasks were incubated for 2 d in a reciprocal shaker (100 rpm, 27 ^{o}C) and were subsequently used to inoculate 100 ml medium (5.5 g l^{-1} yeast extract; 25 g l^{-1} sea salt and 25 g l^{-1} glucose) in 500-ml erlenmeyer flasks or shake-flask experiments. After incubation in a reciprocal shaker for 3 d, these cultures were used to inoculate bioreactors (see below). Shake-flask experiments were performed in 300 ml flasks with 50 ml medium (2 g l^{-1} yeast extract; 25 g l^{-1} sea salt and 0-25 g l^{-1} ethanol). These flasks were incubated for 3 d in a reciprocal shaker (100 rpm, 27 ^{o}C). Samples (0.5-1 ml) were taken twice daily for optical density measurements at 470 nm.

Cultivation in bioreactors

Fed-batch cultivation was performed at 27 ^{o}C in 2-l laboratory bioreactors (Applikon, Schiedam, The Netherlands) controlled by the computer program BioXpert and an Applicon ADI-1020 biocontroller. The dissolved oxygen tension (DOT) was kept above 30% of air saturation by automatically controlling the stirrer speed (range: 200-1250 rpm) and by flushing (1 l min^{-1}) with filter-sterilised air. The stirrer speed was programmed only to increase and not to decrease. Foam production was repressed by addition of 4 droplets of MAZU DF 8005 antifoam (BASF, Cheadle, UK). The initial medium (1 l) contained 10 g l^{-1} yeast extract; 25 g l^{-1} sea salt, 5.5 g l^{-1} ethanol, 0.5 g l^{-1} Glucanex ® (Novo Nordisk, Neumatt, Switzerland) and a 10% (v/v) inoculum. Yeast extract and Glucanex were filter-sterilised. Ethanol feeding was automatically initiated when, after 24-29 h of cultivation, the DOT rose to above 60% of air saturation, indicating ethanol depletion. The initial ethanol feed rate was initially 0.9 and maximally 3.0 g l^{-1} h^{-1}. The feed rate was increased when the DOT decreased after a small manual pulse of ethanol. Throughout cultivation, growth inhibition due to ethanol accumulation was prevented by temporarily and automatically halting the ethanol feed whenever the DOT rose above 35%. The pH was kept at 6.5 ± 0.1 by automated addition of 2M HCl or 0.5 M NaOH.

Analysis of biomass concentration and lipid analysis

Culture samples of 20-40 ml were centrifuged (17,000 *g*, 10 min). The cell pellet was washed in 25 ml demineralised water, lyophilised and weighed in order to determine the biomass concentration. Yield calculations were estimated after a correction for sampling. However, accurate yield calculations would require the use of larger bioreactors, in which sampling has a smaller effect on culture volume. For lipid extraction, lyophilised biomass (100 mg) was incubated for 24 h in 5 ml extraction solution (chloroform:methanol=2:1, containing 0.5 mg butylated hydroxytoluene and, as an internal standard, 1 mg ml^{-1} methyl docosanoate) at room temperature under gentle stirring. The solution with the extracted lipids was separated from the cell debris by centrifugation (1500 *g*, 5 min). To the cell debris another 3 ml extraction solution was added and incubated for another 20 h. The solution with the extracted lipids was again separated from the cell debris by centrifugation and pooled with the solution of the first

incubation. Methylated fatty acids were prepared from the lipid extract with trimethylsulphonium hydroxide (Butte 1983) and analysed by gas chromatography as previously described (de Swaaf et al. 1999). Values for the lipid content of the biomass and DHA content of the lipid are expressed in weight percentages.

Results

Batch growth of *C. cohnii* with ethanol as carbon source

The influence of ethanol concentration on growth of *C. cohnii* was studied in shake-flask cultures grown on a complex medium containing yeast extract and sea salt (de Swaaf et al. 1999). Growth on yeast extract as the sole carbon source was negligible (Fig. 1). Growth did occur when ethanol was added at a concentration of 5 or 10 g l^{-1} (Fig. 1). The specific growth rates at these ethanol concentrations, calculated from the exponential part of the growth curves, was 0.05 h^{-1}. In contrast to the cultures grown on 5 g l^{-1} ethanol, cultures grown on 10 g l^{-1} ethanol exhibited a significant lag phase. This, together with the observation that no growth occurred at ethanol concentrations of 15 g l^{-1} and above, indicated that higher ethanol concentrations inhibit growth of *C. cohnii*.

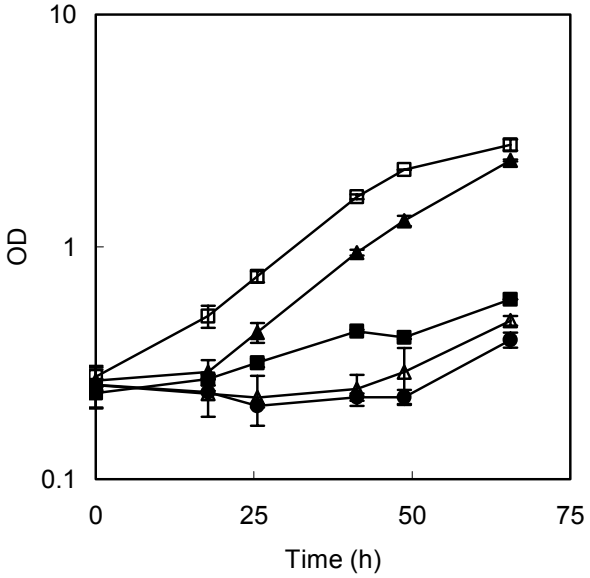

Fig. 1 Influence of initial ethanol concentration on growth of *C. cohnii* in shake-flask cultures. The medium contained: 2 g l^{-1} yeast extract; 25 g l^{-1} sea salt; 10% (v/v) inoculum and was supplemented with ethanol at concentrations of 0 (■), 5 (□), 10 (▲), 15 (Δ) or 25 (●) g l^{-1}. Data are represented as the average ± deviation of two independent replicate cultures.

Controlled feeding of ethanol to *C. cohnii* cultures

The ethanol sensitivity of *C. cohnii*, as revealed by the shake-flask experiments (Fig. 1) showed that batch cultivation is not suitable for high-cell-density cultivation of this alga on ethanol. Therefore, to investigate the potential of ethanol as a carbon substrate for the production of total lipid and DHA, a fed-batch cultivation strategy was developed. The composition of the complex medium was based on earlier studies on glucose- and acetic acid-grown fed-batch cultivations of this alga (de Swaaf et al. 2003). The polysaccharide-hydrolysing enzyme preparation Glucanex was added to the cultivation media media to decrease culture viscosity, thereby facilitating oxygen transfer (de Swaaf et al. 2003).

Pilot experiments identified the dissolved oxygen tension (DOT) in the cultures as a reliable input signal for automatic control of the ethanol feed. Depletion of ethanol in batch cultures readily became apparent by a fast increase of the DOT from ca. 30% to >60% of air saturation. This fast increase of the DOT was used as a signal to automatically initiate the ethanol feed after the batch phase. During the fed-batch phase, growth inhibition by ethanol (Fig. 1) became evident from a slow increase of the DOT. When the ethanol feed was interrupted at the first sign of overfeeding, the culture quickly recovered and consumed the accumulated ethanol. In practice, the ethanol feed was interrupted when the DOT increased to above 35% of air saturation and was only resumed when a fast increase of the DOT to above 60% of air saturation occurred.

Fed-batch cultivation on ethanol

Based on several preliminary experiments, fed-batch cultivation of *C. cohnii* was performed with the feed-control strategy outlined above, using an initial feed rate of 0.9 g ethanol l^{-1} h^{-1}. In a representative ethanol-grown fed-batch cultivation of *C. cohnii*, 300 g ethanol was added over a total fermentation time of 220 h (Fig. 2A). The estimated specific growth rate over the first 52 h of incubation was 0.047 h^{-1}, which is in good agreement with the maximum specific growth rate estimated from ethanol-grown shake-flask cultures (Fig. 1). Between 52 and 146 h, the increase in the total biomass concentration was linear, indicating that growth was limited by a nutrient other than ethanol. After this period, the increase in biomass concentration levelled off (Fig. 2A). The total lipid content of the biomass increased most strongly between 52 and 146 h of

cultivation and reached a maximum of 42% at the end of the process (Fig. 2B). The DHA content of the lipid, which varied between 32 and 44% during the initial phase of the fermentation, remained constant at 33±1% during the final 100 h of the process (Fig. 2B). The volumetric rate of DHA production (r_{DHA}) reached a maximum of 53 mg l^{-1} h^{-1} at 220 h (Fig. 2C). The final concentrations of biomass dry weight, lipid and DHA were 83, 35 and 11.7 g l^{-1}, respectively (Fig. 2A and C). The biomass yield on ethanol, corrected for the samples withdrawn during the fermentation process, was 0.31 g biomass (g ethanol)$^{-1}$.

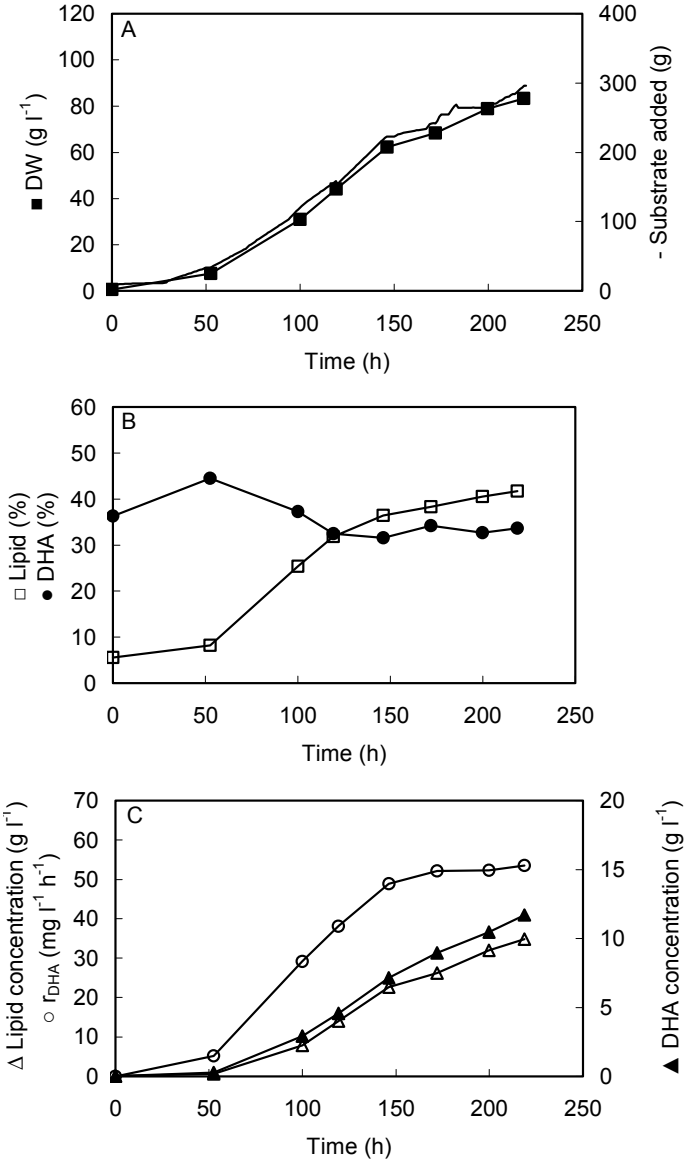

Fig. 2A, B, C Fed-batch cultivation of *C. cohnii* with a feed consisting of pure ethanol. The initial medium contained: 10 g l^{-1} yeast extract; 25 g l^{-1} sea salt; 5.5 g l^{-1} ethanol; 0.5 g l^{-1} Glucanex and 10% (v/v) inoculum.
A: Biomass dry weight (DW) and ethanol added.
B: Lipid content of dry biomass (Lipid), DHA content of lipid (DHA).
C: Lipid concentration, overall volumetric productivity of DHA (rDHA) and DHA concentration. Data from an independent replicate culture differed by less than 5%.

Discussion

The volumetric productivity of DHA (r_{DHA}) is a crucial parameter in commercial DHA production via microbial fermentation (Sijtsma et al. 1998). The r_{DHA} in ethanol-grown fed-batch cultures of *C. cohnii* was much higher than in fed-batch cultures grown on glucose (Table 1), currently the preferred carbon source for DHA production with this alga, and even higher than in acetic acid-grown fed-batch cultures (Table 1). A detailed comparison of data obtained from ethanol-grown fed-batch cultures with previously obtained data from acetic acid-grown fed-batch cultures (Table 1) revealed that the DHA content of the total lipid fraction was virtually the same for the two carbon sources. Although the total lipid content was higher in the acetic acid-grown fed-batch cultures, the faster biomass production in the ethanol-grown cultures resulted in a higher volumetric rate of DHA production (r_{DHA}). These results demonstrate that ethanol is a very promising carbon source for the production of DHA with *C. cohnii*.

Ethanol has several further advantages over acetic acid as a carbon source for industrial fermentation processes. Firstly, the biomass yield of microorganisms on ethanol is generally higher than that on acetic acid (Verduyn et al. 1991; Linton and Rye 1989). This was also the case with *C. cohnii*, as the yields of biomass on ethanol and acetic acid for *C. cohnii* were 0.31 g biomass g^{-1} (this study) and 0.13 biomass g^{-1} (de Swaaf et al. 2003), respectively. This, together with the lower (tax free) price of ethanol compared to that of acetic acid (Tilton 2002) results in better carbon source economy. Yet another advantage is that ethanol is less corrosive than acetic acid (Lide 1997), which may reduce capital investment and maintenance costs for fermentation hardware. On the other hand, the higher flammability of ethanol (Furr 2000) may require special precautions for large-scale fermentation processes. However, substrate flammability has not prevented the implementation of other fermentation processes with methanol as the carbon source (Gellissen 2000).

One of the major constraints in high-cell-density cultivation of *C. cohnii* is culture viscosity. This viscosity is at least partly caused by the production of extracellular polysaccharides and reduces the efficiency of oxygen transfer (de Swaaf et al. 2001). Despite the use of well-mixed laboratory fermenters and the addition of a polysaccharide-hydrolysing enzyme preparation (de Swaaf et al. 2003) the stirrer had to be operated at maximum capacity to maintain aerobic conditions. As efficient gas transfer is more difficult to achieve in large-scale

cultivations than in small-scale laboratory fermenters (Einsele 1978; Oosterhuis and Kossen 1983) this may present an important challenge in scale-up. Selection of *C. cohnii* strains that produce less extracellular polysaccharides may be an important step in further process development.

Table 1 Comparison of glucose (50% w/v)-, ethanol- and acetic acid-grown fed-batch cultures of *C. cohnii*. Selected parameters are shown for time point 120 h (Glucose), 200 h (Ethanol) and 210 h (Acetic acid). Data from glucose- and acetic acid-grown fed-batch cultures of *C. cohnii* was taken from Chapter 4. Values of the ethanol and acetic acid grown cultures represent the average ± deviation of two independent fed-batch cultures. Cultivation conditions of ethanol-grown cultures are given in the Materials and Methods section. Fatty acids other than DHA are indicated as y:x where y represents the number of carbon atoms and x the number of double bonds.

	Feed		
	Glucose 50% (w/v)	Ethanol	Acetic acid
Time (h)	120	200	210
Biomass (g l^{-1})	26	77±3	59±3
Lipid content (% w/w)	15	41±1	50±1
Lipid concentration (g l^{-1})	3.8	31±1	30±1
12:0 in lipid (% w/w)	3	11±0	10±1
14:0 in lipid (% w/w)	18	21±0	25±0
16:0 in lipid (% w/w)	22	15±0	19±1
16:1 in lipid (% w/w)	-	6±0	2±0
18:0 in lipid (% w/w)	3	1±0	1±0
18:1 in lipid (% w/w)	8	13±0	11±0
DHA in lipid (% w/w)	46	33±0	32±0
DHA concentration (g l^{-1})	1.7	10.1±0.4	9.5±0.1
r_{DHA} (mg l^{-1} h^{-1})	14	51.4±2.1	44.5±0.5

Although this study clearly demonstrates the potential of ethanol as a carbon source for large-scale production of DHA, many aspects of the process still need to be optimised. This includes medium composition. Although commonly applied in studies on DHA production by *C. cohnii* (de Swaaf et al. 1999), the yeast-extract based complex medium used in this study is unlikely to be optimally balanced for high-cell-density DHA production. Moreover, carbon-

source feeding strategy and control can be optimised. Instead of a feedback control of the ethanol feed via the dissolved oxygen tension, as applied in this study, various alternative ethanol-feeding strategies may be designed to keep the ethanol concentration in the culture at a defined, optimal concentration throughout the fermentation. Such strategies might be based on liquid-phase or gas-phase ethanol sensors (Noronha et al. 1999) as on-line input for control of the ethanol feed or, alternatively, involve pre-programmed ethanol feed profiles. In addition to optimising process conditions, strain selection may lead to further improvement of the productivity and final concentration of DHA in ethanol-fed *C. cohnii* cultures. A useful starting point for such a strain-selection programme would be a comparison of different wild-type strains of this alga, especially with respect to specific growth rate, r_{DHA} and DHA content during growth on ethanol.

Acknowledgement

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References

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6:123-129

Berg MA van den, Jong-Gubbels P de, Kortland CJ, Dijken JP van, Pronk JT, Steensma HY (1996) The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. J Biol Chem 271:28953-28959

Butte W (1983) Rapid method for the determination of fatty acid profiles from rats and oils using trimethylsulphonium hydroxide for transesterification. J Chromatogr 261:142-145

Einsele A (1978) Scaling up bioreactors. Proc Biochem 13 (7):13-14

Furr AK (editor-in-chief) (2000) CRC Handbook of laboratory safety, 5th edn. CRC Press, Boca Raton London New York Washington D.C.

Gellissen G (2000) Heterologous protein production in methylotrophic yeasts. Appl Microbiol Biotechnol 54:741-750

Harrington GW, Holz GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochim Biophys Acta 164:137-139

Hornstra G (2000) Essential fatty acids in mothers and their neonates. Am J Clin Nutr 71:1262-1269

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. Lipid Technol 8:107-110

Lide DR (editor-in-chief) (1997) CRC Handbook of chemistry and physics. CRC Press, Boca Raton New York

Linton JD, Rye AJ (1989) The relationship between the energetic efficiency in different micro-organisms and the rate and type of metabolite overproduced. J Ind Microbiol 4:85-96

Martinez-Blanco H, Reglero A, Fernandez-Valverde M, Ferrero MA, Moreno MA, Penalva MA, Luengo JM (1992) Isolation and characterization of the acetyl-CoA synthetase from *Penicillium chrysogenum*. Involvement of this enzyme in the biosynthesis of penicillins. J Biol Chem 267:5474-5481

Nettleton JA (1993) Are *n*-3 fatty acids essential nutrients for fetal and infant development? J Am Diet Assoc 93:58-64

Noronha SB, Wagner LW, Matheson NH, Shiloach J (1999) Use of an ethanol sensor for feedback control of growth and expression of TBV25H in *Saccharomyces cerevisiae*. Biotechnol Bioeng 63:285-289

Oosterhuis NMG, Kossen NWF (1983) Oxygen transfer in a production scale bioreactor. Chem Eng Res Des 61:308-312

Provasoli L, Gold K (1962) Nutrition of the american strain of *Gyrodinium cohnii*. Arch Mikrobiol 42:196-203

Ratledge C, Kanagachandran K, Anderson AJ, Grantham DJ, Stephenson JC (2001) Production of docosahexaenoic acid (DHA) by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. Lipids 36:1241-1246

Sijtsma L, Springer J, Meesters PAEP, Swaaf ME de, Eggink G (1998) Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae. Recent Res Dev Microbiol 2:219-232

Swaaf ME de, Rijk TC de, Eggink G, Sijtsma L (1999) Optimisation of docosahexaenoic acid production in batch cultivations by *Crypthecodinium cohnii*. J Biotechnol 70:185-192

Swaaf ME de, Grobben GJ, Eggink G, Rijk TC de, Meer P van der, Sijtsma L (2001) Characterisation of extracellular polysaccharides produced by *Crypthecodinium cohnii*. Appl Microbiol Biotechnol 57:395-400

Swaaf ME de, Sijtsma L, Pronk JT (2003) High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*. Biotechnol Bioeng 81:666-672

Tilton H (editor-in-chief) (2002) List of chemical prices. Chemical Market Reporter (Januari 14):16-19

Verduyn C, Stouthamer AH, Scheffers WA, van Dijken JP (1991) A theoretical evaluation of growth yields of yeasts. Ant van Leeuwenhoek Int J Gen and Mol Microbiol 59:49-63

Woodward J, Merrett MJ (1975) Induction potential for glyoxylate cycle enzymes during the cell cycle of *Euglena gracilis*. Eur J Biochem 15:555-559

Chapter 7

Analysis of docosahexaenoic acid biosynthesis in *Crypthecodinium cohnii* by ^{13}C labelling and desaturase inhibitor experiments

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The lipids of the heterotrophic microalga *Crypthecodinium cohnii* contain the ω-3 polyunsaturated fatty acid, docosahexaenoic acid (22:6) to a level of over 30%. The pathway of 22:6 synthesis in *C. cohnii* is unknown. The ability of *C. cohnii* to use ^{13}C labelled externally supplied precursor molecules for 22:6 biosynthesis was tested by ^{13}C-NMR analysis. Furthermore, the presence of desaturases (typical for aerobic polyunsaturated fatty acid synthesis) was studied by the addition of desaturase inhibitors in the growth medium. The addition of 1-^{13}C acetate or 1-^{13}C butyrate in the growth medium resulted in 22:6 with only the odd carbon atoms enriched. Apparently, two-carbon atom units were used as building blocks for 22:6 synthesis and butyrate was first split into two-carbon atom units prior to incorporation in 22:6. When 1-^{13}C oleic acid was added to the growth medium, 1-^{13}C oleic acid was incorporated into the lipids of *C. cohnii* but was not used as a precursor for the synthesis of 22:6. Desaturase inhibitors (norflurazon and propyl gallate) inhibited lipid accumulation in *C. cohnii*. The fatty acid profile, however, was not altered. In contrast, in the arachidonic acid producing fungus *Mortierella alpina* these inhibitors not only decreased the lipid content but also altered the fatty acid profile. Our results can be explained by the presence of three tightly regulated separate systems for the fatty acid production by *C. cohnii*, namely for (1) the biosynthesis of saturated fatty acids, (2) the conversion of saturated fatty acids to monounsaturated fatty acids, and (3) the *de novo* synthesis of 22:6 with desaturases involved.

Introduction

The family of ω-3 polyunsaturated fatty acids (PUFAs) includes α-linolenic acid (18:3), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). Especially 22:6 is recognised to have a beneficial influence on human health (Nettleton 1993; Hornstra 2000) and is included in various infant foods (Barclay et al. 1994; Ratledge 2001).

Long-chain PUFAs (containing 20 or more carbon atoms and over 2 double bounds) are widespread in the marine food chain. The primary producers are marine algae. Despite the importance of these PUFAs their pathways of formation are not well known.

The chloroplastless heterotrophic marine microalga *Crypthecodinium cohnii* is commercially used to produce an oil rich in 22:6 (Kyle 1996) and has been used to study the pathways involved in PUFA biosynthesis by marine algae (Beach et al. 1974; Sonnenborn and Kunau 1982; Henderson and Mackinlay 1991). *C. cohnii* can accumulate lipid to over 20% of dry weight with a high content of 22:6 (over 30% of the total lipid). Other PUFAs remain below 1% of the total fatty acid content (Harrington and Holz 1968; Beach and Holz 1973; de Swaaf et al. 1999). This is remarkable as most marine microalgae rich in PUFAs contain intermediate fatty acids in the cascade of elongation and desaturation (Wood 1974; Viso and Marty 1993; Servel et al. 1994).

In *C. cohnii*, a fatty acid synthetase (FAS) system is operative. Sonnenborn and Kunau (1982) reported that a purified cytosolic enzyme complex of *C. cohnii* produced (*in vitro*) saturated fatty acids, mainly 14:0 and 16:0. Possibly, the FAS system supplies the precursors for the 22:6 biosynthesis. In order to substantiate this, Beach et al. (1974) incubated *C. cohnii* cells in the presence of ^{14}C labelled fatty acids ranging from 10 to 18 carbon atoms. In all incubations label was detected in oleic acid (Δ9 18:1 or in short 18:1, the most abundant monounsaturated fatty acid in *C. cohnii*) but not in 22:6. In contrast, Henderson and Mackinlay (1991) detected a small amount of label in 22:6 when the alga was grown in the presence of ^{14}C labelled 18:0 or 18:1. A clarification whether this amount originated from subsequent elongation and desaturation of the precursors or from β-oxidation and *de novo* synthesis of 22:6 was not evident but may be important for the elucidation of the pathway of 22:6 formation.

In order to test the ability of *C. cohnii* to use precursor molecules for 22:6 biosynthesis, in this study *C. cohnii* was grown in the presence of ^{13}C labelled short- and long-chain organic acids

and the biosynthetic products were analysed using ^{13}C-NMR. Furthermore, the overall positional distribution of 22:6 in the lipids was determined.

In addition, the presence of desaturases (typical for aerobic PUFA synthesis) was studied by the addition of desaturase inhibitors in the growth medium of *C. cohnii*. Apart from the presence of high levels of 22:6 in *C. cohnii*, so far evidence for the presence (or absence) of desaturases involved in 22:6 biosynthesis in *C. cohnii* is absent in literature. Previously, it was shown that norflurazon (a herbicide) inhibited the Δ6 desaturase, the carotenoid biosynthesis and the chlorophyll accumulation in the microalgae *Spirulina platensis* and *Monodus subterraneus* (Cohen and Heimer 1990). Propyl gallate, which is used as an antioxidant in food applications, inhibited Δ5 and Δ6 desaturases in both rat liver microsomes and the arachidonic acid (ω-6 20:4)-producing fungus *Mortierella alpina* (Kawashima et al. 1996). Acrylic acid inhibited the enzymes acyl-CoA synthetase and 3-ketoacyl-CoA thiolase (Timm and Steinbüchel 1990) in *Pseudomonas aeruginosa*. We have studied the influence of norflurazon, propyl gallate and acrylic acid on the fatty acid production and composition of *C. cohnii* and *M. alpina*.

Materials and methods

Media, maintenance of cells and cultivation conditions

C. cohnii (ATCC 30772) cells were grown in static cultures (50 ml in 250 ml flasks) at 25-27 ^{o}C in the dark. The static cultures were subcultured within 4 weeks for maintenance or used after growth for 4-10 days as inoculum for precultures. Unless indicated otherwise, the standard growth medium of *C. cohnii* contained 9 g l^{-1} glucose; 2 g l^{-1} yeast extract (Oxoid, Basingstoke, UK); 25 g l^{-1} sea salt (Sigma-Aldrich, Zwijndrecht, The Netherlands) and a 10% (v/v) inoculum. All medium components were heat-sterilised (121 ^{o}C) separately.

Shake-flask cultivations of *C. cohnii* (precultures and shake-flask experiments), containing 50 ml medium in 250 ml flasks, were carried out at 27 ^{o}C, pH 6.5 and 100 rpm in a reciprocal shaker. The precultures for shake-flask experiments were incubated for 3 days. In shake-flask experiments the cells were harvested after 2 days of incubation.

For ^{13}C labelling studies, *C. cohnii* was grown on standard growth medium supplemented with 0.4 g l^{-1} 1-^{13}C oleic acid (ARC laboratories, Amsterdam, The Netherlands) or with 0.4 g l^{-1}

unlabelled oleic acid. In addition, *C. cohnii* was grown on 1 g l^{-1} yeast extract; 25 g l^{-1} sea salt salt; a 10% inoculum and as a carbon source either 6.2 g l^{-1} 1-^{13}C sodium acetate (ARC laboratories) or 5 g l^{-1} 1-^{13}C sodium butyrate (ARC laboratories).

In experiments with inhibitors, *C. cohnii* was grown on 9 g l^{-1} glucose; 1 g l^{-1} yeast extract; 25 g l^{-1} sea salt and a 10% inoculum supplemented with either no inhibitor, 0.1 g l^{-1} norflurazon (Novartis, Basel, Switserland), 0.1 g l^{-1} propyl gallate (Sigma-Aldrich) or 0.1 g l^{-1} acrylic acid (Sigma-Aldrich). Norflurazon was supplied to the medium by adding 0.5 ml of 10 g l^{-1} norflurazon in DMSO.

Mortierella alpina (ATCC 32222) cells were maintained on YPD agar (Duchefa, Haarlem, The Netherlands) plates at 25 ^{o}C. From 1-3 weeks old agar plates, 5 times 1 cm^2 *M. alpina* mycelium was cut and used to inoculate 50 ml YPD (Duchefa) medium in 300 ml flasks and statically incubated at 25 ^{o}C. After 2 weeks incubation the cultivation broth was treated in a Waring commercial blender (Cole-Parmer, Vernon Hills, Illinois, USA) for 20 s at 18,000 rpm. The resulting broth was used to inoculate shake-flask experiments with *M. alpina*.

Shake-flask cultivations of *M. alpina*, containing 25 ml medium in 100 ml flasks, were carried out at 30 ^{o}C, pH 6.0 and 250 rpm in a rotary shaker. The growth medium contained 20 g l^{-1} glucose; 5 g l^{-1} yeast extract; a 10% (v/v) inoculum and either no inhibitor, 0.1 g l^{-1} norflurazon, 0.1 g l^{-1} propyl gallate or 0.1 g l^{-1} acrylic acid. Norflurazon was supplied to the medium by adding 0.25 ml of 10 g l^{-1} norflurazon in DMSO. Cells were harvested after 4 days incubation.

Lipid analysis

Culture samples of 25-50 ml were centrifuged (1,500 *g* for 5 min). The cell pellet was washed in 25 ml demineralised water, lyophilised and weighed in order to determine the biomass concentration. Prior to and after lyophilisation, the samples were stored at –20 °C.

The oil was extracted from lyophilised cells by a modification of the method of Bligh and Dyer (1959). Lyophilised cells (100 mg or more) were weighed accurately into a 10 ml centrifuge tube. For extraction, 5 ml chloroform/methanol (2/1) containing 1.0 mg ml^{-1} of an internal standard (see below) and 0.5 mg ml^{-1} butylated hydroxytoluene was used and the tube was shaken gently overnight. As internal standards, methyl docosanoate (22:0) was used in extractions of *C. cohnii*-biomass whereas methyl pentadecanoate (15:0) was used in extractions

of *M. alpina*-biomass. After centrifugation at 1,500 *g* for 5 minutes, the supernatant containing the extracted oil was stored at 4 °C until analysis.

Methyl esters of the fatty acids present in the Bligh-Dyer extracts were prepared with trimethylsulphonium hydroxide according to Butte (1983) and analysed by gas chromatography as previously described (de Swaaf et al. 1999). Values for the lipid content of the biomass and DHA content of the lipid are expressed in weight percentages.

Lipid fractionation

Polar lipid fractions were prepared from extracted oils by thin layer chromatochraphy on Silicagel-G plates (Merck, Darmstadt, Germany) using a hexane/diethylether/acetic acid (70/30/1, v/v/v) solution (Bell and Henderson 1990). Peaks were made visible under UV-light by spraying with 2,7-dichlorofluorescein (0.2% (w/v) in ethanol). The polar lipid fractions were scraped off and extracted overnight with 3.0 ml chloroform/methanol (2/1) containing 1.0 mg ml^{-1} methyl docosanoate and 0.5 mg ml^{-1} butylated hydroxytoluene. After centrifugation at 1,500 *g* for 5 minutes, the supernatant was stored at 4 °C prior to derivatization, GC and GC-MS analysis. Methyl esters were prepared according to Butte (1983).

GC-MS was performed on an equal column and with an equal temperature program as previously described (de Swaaf et al. 1999). The MS was operated at 200°C in the SCAN mode to check the identity of the components (25-500 D sec^{-1}) after which quantification was performed in the selected ion recording (SIR) mode. The SIR mode was used to enhance the selectivity and lower the detection limit. The ions m/z 74 and 75 containing the first and second C-atom of a methyl ester (McLafferty and Tureček 1993) were chosen to calculate the enrichment factor.

^{13}C-NMR spectroscopy

^{13}C-NMR spectra were recorded on a Bruker AMX400-wb spectrometer (Billerica, USA), operating at 100.62 MHz. Samples (approximately 50 mg ml^{-1}) were dissolved in deuterated chloroform with tetramethylsilane as internal standard. The free induction decay (FID) was acquired at ambient temperature using a sweep width of 300 ppm, 32k time domain points and

unless indicated otherwise a 60 s pulse repetition time, using an inverse-gated proton decoupled technique. Typically 3333 scans were collected using a 90˚ excitation pulse of 6.4 μs.

In quantitative ^{13}C-NMR of the lipid extracts from *C. cohnii* cells grown on 1-^{13}C acetate and 1-^{13}C butyrate both the Nuclear Overhauser Effect (NOE) and the relaxation time T_1 must be taken into account. The NOE, causing intensity differences between primary, secondary, tertiary, and quaternary carbon atoms, was suppressed by using an inverse-gated proton decoupling technique. The effect caused by differences in T_1 value (0.29-6.15 s according to Aursand et al. 1993) was neutralised by using a pulse repetition time of 60 s. In ^{13}C-NMR spectra of lipid extracts of *C. cohnii* cells grown in the presence of oleic acid and 1-^{13}C oleic acid a pulse repetition time of 15 s was applied.

Results

^{13}C labelling of 22:6

Small organic acids like acetate, propionate and butyrate can be used as primary carbon sources by *C. cohnii* (data not shown). Whether small organic acids (other than acetate) can be used as precursors in the fatty acid biosynthesis by *C. cohnii* is not known. Most likely, the basic building block of fatty acid biosynthesis in *C. cohnii* is an activated acetate (two-carbon atom) unit, acetyl-CoA (Sonnenborn and Kunau 1982). To substantiate this, *C. cohnii* cells were grown on 1-^{13}C acetate and 1-^{13}C butyrate as carbon sources and the resulting lipids were extracted and analysed by ^{13}C-NMR.

With 1-^{13}C acetate in the growth medium only the odd carbon atoms of 22:6 were enriched (Fig. 1). Similar results were found when *C. cohnii* was grown on 1-^{13}C butyrate; the four-carbon atom unit did not serve as a direct precursor for 22:6 biosynthesis but was degraded to two-carbon atom units. These findings further demonstrate that a two-carbon atom unit is the building block for 22:6 biosynthesis in *C. cohnii*.

^{13}C-NMR spectra of PUFA-containing lipids enable the determination of the positional distribution of the PUFAs in the lipids (Aursand et al. 1993). The positional distribution of 22:6 and the other fatty acids in the lipids was similar with both carbon sources. The location of 22:6

in the lipids was 60% *sn*(1+3) and 40% *sn*2. Other fatty acids were located for 70% at the *sn*(1+3)- and for 30% at the *sn*2-position of the lipids.

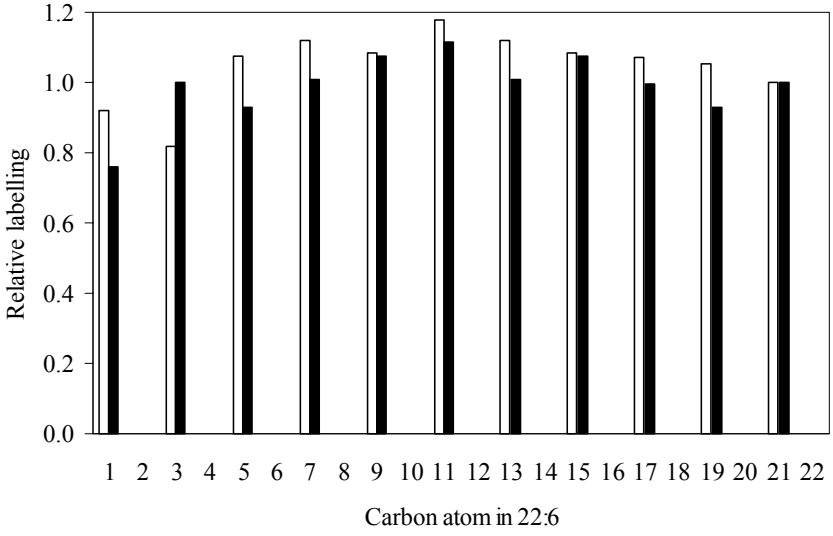

Fig. 1 Relative ^{13}C labelling of the carbon atoms of 22:6 produced by *C. cohnii* cells grown for 2 days on 6.2 g l^{-1} 1-^{13}C acetate (white bars) and 5 g l^{-1} 1-^{13}C butyrate (black bars). Other medium components included 1 g l^{-1} yeast extract; 25 g l^{-1} sea salt and a 10% inoculum. Data were normalised against the intensities of carbon atom 21 of 22:6.

A demonstration of the ability of *C. cohnii* to use externally supplied fatty acids like oleic acid (18:1) as a precursor for 22:6 would provide important information for the elucidation of the unknown pathway of 22:6 synthesis. If *C. cohnii* is able to use 18:1 as a precursor for 22:6, the label of 1-^{13}C 18:1 is then expected to appear on carbon atom 5 (C5) as counted from the carboxyl terminus in 22:6.

Lipid extracts from cultivations of *C. cohnii* in the presence of 1-^{13}C 18:1 were prepared and analysed with ^{13}C-NMR. From the large peak (not shown) in the ^{13}C-NMR spectra corresponding to C1 of fatty acyl groups other than 22:6 it could be concluded that the externally supplied 1-^{13}C 18:1 was taken up by *C. cohnii* and incorporated into the lipids. Furthermore, GC-MS analysis of the lipid fraction containing the phospholipids (the polar fraction) showed that 1-^{13}C 18:1 was incorporated into the phospholipids; the enrichment factor was about 14. The fatty acids linked to phospholipids are thought to serve as precursors for the production of 22:6 (Bell and Henderson 1990). However, as the intensity of the signal corresponding to C5 of 22:6 in the lipid extract was not significantly higher than other carbon atoms in 22:6 (like C4; Fig. 2) it is concluded that no significant elongation and desaturation of 1-^{13}C 18:1 to 22:6 had occurred.

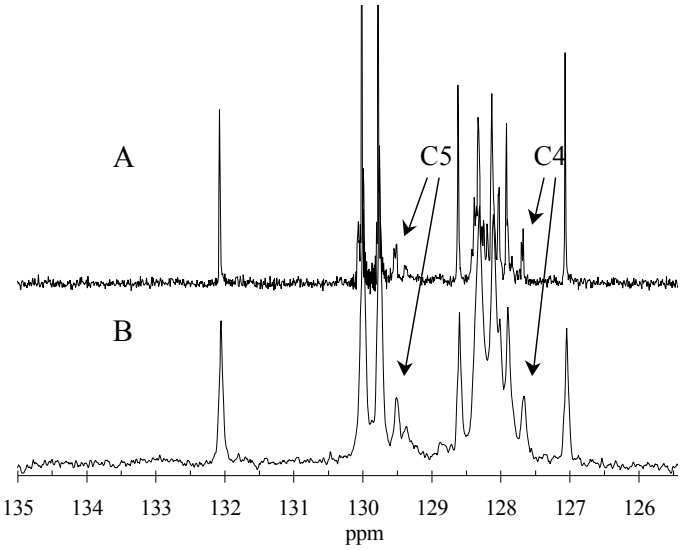

Fig. 2 ^{13}C-NMR spectra of lipid extracts from *C. cohnii* cells grown for 2 days in the presence of (A) 0.4 g l^{-1} 1-^{13}C oleic acid and (B) 0.4 g l^{-1} oleic acid. Other medium components included 9 g l^{-1} glucose; 2 g l^{-1} yeast extract; 25 g l^{-1} sea salt and a 10% inoculum. C4 and C5 indicate the chemical shift positions of carbon atoms 4 and 5 of 22:6, respectively.

Addition of inhibitors into the growth media

The activity of desaturases and other enzymes involved in the fatty acid synthesis by microorganisms can be decreased by the addition of inhibitors into the growth medium. Fatty acid analysis of the resulting biomass can give an indication of the pathways involved in fatty acid formation and can indicate the presence (or absence) of desaturases in *C. cohnii*.

The influences of the inhibitors norflurazon, propyl gallate and acrylic acid on the lipid and fatty acid content of *C. cohnii* and, as a control, *M. alpina* were compared. The pathway of arachidonic acid (ω-6 20:4) biosynthesis in *M. alpina* has been reviewed by Ratledge (2001) and is relatively well known as compared to the route of 22:6 biosynthesis in *C. cohnii*. By a cascade of desaturations (DS) and an elongation (EL) arachidonic acid is formed from 18:0 in *M. alpina* (18:0 $^{\Delta 9\ DS}\rightarrow$ Δ9 18:1 $^{\Delta 12\ DS}\rightarrow$ ω-6 18:2 $^{\Delta 6\ DS}\rightarrow$ ω-6 18:3 $^{EL}\rightarrow$ ω-6 20:3 $^{\Delta 5\ DS}\rightarrow$ ω-6 20:4).

During growth of *M. alpina* for 4 days on a medium of 5 g l^{-1} yeast extract; 20 g l^{-1} glucose and a 10% (v/v) inoculum, 11 g l^{-1} biomass was produced with a lipid content of 30% and an arachidonic acid content of the lipid of 41% (Table 1). Inclusion of 0.1 g l^{-1} norflurazon, propyl gallate and acrylic acid in the growth medium resulted in decreased final biomass and lipid concentrations in all three cases.

The influence on the fatty acid profiles of *M. alpina* depended on the inhibitor used (Table 1). Norflurazon strongly decreased the 20:4 percentage of the lipid in *M. alpina* whereas the percentages of 18:1 and 16:0 increased. Propyl gallate in the medium resulted also in a strong

decrease of 20:4 in the lipid. The fatty acids that increased were 18:2, 18:1 and 16:0. The fatty acid profile of *M. alpina* was least affected by the inclusion of acrylic acid in the medium.

Table 1 Influence of norflurazon (norf), propyl gallate (prop) and acrylic acid (acryl) on biomass, cellular lipid content and fatty acid composition of *M. alpina* after 4 days incubation. The initial medium contained 5 g l^{-1} yeast extract; 20 g l^{-1} glucose; 10% inoculum (v/v) and was supplemented with either no inhibitor, 0.1 g l^{-1} norf, 0.1 g l^{-1} prop or 0.1 g l^{-1} acryl. Data points are average values of duplicate cultivations. Lipid content of the cells (Lipid) and fatty acids of the lipid are expressed in percentages. Fatty acids are indicated as y:x where y represents the number of carbon atoms and x the number of double bonds. 18:1 stands for oleic acid (Δ9 18:1) and 18:2, 18:3, 20:3 and 20:4 are ω-6 PUFAs.

Inhibitor	Biomass (g l^{-1})	Lipid (%)	14:0 (%)	16:0 (%)	18:0 (%)	18:1 (%)	18:2 (%)	18:3 (%)	20:0 (%)	20:3 (%)	20:4 (%)	22:0 (%)
-	11	30	1	12	14	17	6	5	1	4	41	1
Norf	8.7	18	2	18	13	31	7	5	1	3	21	1
Prop	9.4	15	1	22	13	28	13	3	1	4	15	2
Acryl	8.0	18	1	17	13	20	9	4	1	4	31	1

Table 2 Influence of norflurazon (norf), propyl gallate (prop) and acrylic acid (acryl) on biomass, cellular lipid content and fatty acid composition of *C. cohnii* after 2 days incubation. The initial medium contained 1 g l^{-1} yeast extract; 25 g l^{-1} sea salt; 9 g l^{-1} glucose; 10% inoculum (v/v) and was supplemented with either no inhibitor, 0.1 g l^{-1} norf, 0.1 g l^{-1} prop or 0.1 g l^{-1} acryl. Data points are average values of duplicate cultivations. Lipid content of the cells (Lipid) and fatty acids of the lipid are expressed in percentages. Fatty acids are indicated as y:x where y represents the number of carbon atoms and x the number of double bonds. The position of the double bond in 16:1 and 18:1 is at the Δ9 position.

Inhibitor	Biomass (g l^{-1})	Lipid (%)	12:0 (%)	14:0 (%)	16:0 (%)	16:1 (%)	18:0 (%)	18:1 (%)	22:6 (%)
-	2.7	20	7	24	24	1	2	11	32
Norf	1.3	16	6	22	24	1	2	11	35
Prop	1.7	9	3	23	27	1	4	10	32
Acryl	2.6	18	6	24	26	1	3	9	32

During growth of *C. cohnii* for 2 days on a medium of 1 g l^{-1} yeast extract, 9 g l^{-1} glucose, 25 g l^{-1} sea salt and a 10% (v/v) inoculum, 2.7 g l^{-1} biomass was produced with a lipid content of 20% and a 22:6 content of the lipid of 32% (Table 2). Norflurazon and propyl gallate inhibited

both the growth and the lipid accumulation of *C. cohnii* but not in a similar manner (Table 2). Inclusion of norflurazone in the medium decreased biomass production by 50% and the cellular lipid content by 20%. The use of propyl gallate decreased the biomass by 35% but the cellular lipid content by as much as 57%. Apparently, propyl gallate and, to a lesser extent, norflurazon are potent inhibitors for the lipid production in *C. cohnii.* The presence of acrylic acid did not result in a significantly decreased biomass concentration and the lipid content was slightly decreased. Interestingly, in contrast to *M. alpina*, the fatty acid composition of *C. cohnii* was hardly affected upon treatment with all used inhibitors.

Discussion

The positional distribution of 22:6 in lipids is of importance for their nutraceutical or pharmaceutical value. In rats, for instance, the intramolecular PUFA-distribution in the triacylglycerols of tissue (Leray et al. 1993) and lipoproteins (Christensen and Høy 1996) resembled the triacylglycerols of the diet.

We found a uniform intramolecular distribution of 22:6 in the lipids of *C. cohnii* cells by non-destructive ^{13}C-NMR analysis of the lipids. In contrast, by using a method based on digestion of the oil with a positional specific lipase, a strong positional preference of 22:6 in triacylglycerols of *C. cohnii* for *sn*(1+3) was reported previously (Kyle et al. 1992).

^{13}C labels from both 1-^{13}C acetate and 1-^{13}C butyrate were incorporated regularly into 22:6. All odd carbon atoms were enriched and the even carbon atoms were not. It can be anticipated that the labelling of 22:6 is dependent on the selection of the labels present in the carbon source e.g. 2-^{13}C or 2- ^{14}C acetate (or acetic acid) would lead to 22:6 with all even carbon atoms enriched. ^{13}C or ^{14}C labelled varieties of 22:6 may be helpful in nutritional and pharmaceutical studies. With a recently described novel process to produce 22:6 by acetic acid-grown fed-batch cultivation of *C. cohnii* (Ratledge et al. 2001; de Swaaf et al. 2003) an oil containing labelled 22:6 can be produced relatively simply and cheap.

In *C. cohnii* at least three systems appear to be involved in the biosynthesis of the various fatty acids. Systems exist for (1) the production of saturated fatty acids, (2) the conversion of saturated to monounsaturated fatty acids and (3) the production of 22:6.

The saturated fatty acids of *C. cohnii* can be produced by a cytosolic FAS system. A particle free cell extract and a purified FAS system of *C. cohnii* produced only saturated fatty acids (Sonnenborn and Kunau 1982). Assuming all required components were present in the assay, this indicated that in *C. cohnii* the unsaturated fatty acid biosynthesis is dependent on particles like membranes or specific organelles.

Fatty acids added to the growth medium can be elongated to larger saturated fatty acids and be converted to 18:1 but not to 22:6 (this study; Beach et al. 1974; Henderson and Mackinlay 1991). Elongation of added fatty acids, most likely by the action of the FAS system, can thus be followed by desaturation of 18:0 to 18:1 (by a Δ9 desaturase) in *C. cohnii*. The system involved in the conversion of saturated to monounsaturated fatty acids must therefore be accessible for the products of the FAS system. This may be a membrane-associated system located in connection with the cytosol. Desaturation of fatty acids by membrane-associated desaturases and soluble desaturases have been reviewed by Shanklin and Cahoon (1998).

Henderson and Mackinlay (1991) detected a small amount of label in 22:6 when radiolabelled 18:0 and 18:1 were added to the growth medium. This observation was most likely caused by β-oxidation and *de novo* synthesis as, by looking specifically at the labelling of individual carbon atoms in 22:6, we did not observe conversion of 1-^{13}C 18:1 to 22:6. Also the small organic acid butyrate was not used as precursor for 22:6 but was first split into a two-carbon atom unit prior to incorporation in 22:6. The system in *C. cohnii* responsible for 22:6 production appears to synthesise 22:6 only *de novo* with a two-carbon atom unit as the basic building block. This system appears to be compartmentalised from the systems involved in the biosynthesis of saturated and monounsaturated fatty acids and may be located inside an organelle. Examples of compartmentation of the biosynthesis of PUFAs are given in a review by Tocher et al. (1998). In higher plant cells, desaturases operate in the endoplasmatic reticulum and in the chloroplast both as soluble and as membrane-associated desaturases. The fatty acid-desaturation reactions in animals occur in the endoplasmatic reticulum and are catalysed by membrane-associated desaturases. In marine algae, the role of the various organelles in PUFA-biosynthesis is not well known.

The introduction of double bonds in fatty acid biosynthesis can be dependent on oxygen (Ratledge 2001) and can, as suggested by Metz et al. (2001), be a result of anaerobic processes. Such an anaerobic system, however, is unlikely to operate in *C. cohnii* as, in a study of Beach and Holz (1973) the 22:6 and monounsaturated fatty acid content of the lipids sharply decreased when a growing culture of *C. cohnii* was switched from gassing with air to nitrogen gas.

However, whether (oxygen dependent) desaturases are active in the biosynthesis of 22:6 by *C. cohnii* has never been established. The addition of desaturase inhibitors in growth media may alter the fatty acid profile of PUFA-containing microorganisms or decrease lipid production and can thereby give clues about the presence of desaturases and/or the route of PUFA formation.

Control experiments with desaturase inhibitors showed that norflurazon and propyl gallate strongly affected 20:4 biosynthesis in *M. alpina*. Like Kawashima et al. (1996) we measured that upon addition of propyl gallate the percentage of 20:4 decreased and the percentages 16:0, 18:1 and 18:2 increased. Inclusion of norflurazon in the medium resulted in a decrease of 20:4 and increases of 16:0 and 18:1. These findings are in agreement with earlier reports that both norflurazon (Cohen and Heimer 1990) and propyl gallate (Kawashima et al. 1996) can act as desaturase inhibitors. In addition, we showed that the inhibitors have a different mode of action on fatty acid biosynthesis in *M. alpina*.

Both norflurazon and propyl gallate acted differently on *C. cohnii* than on *M. alpina*. The amount of cellular lipid of *C. cohnii* decreased whereas the fatty acid composition of the lipid remained unaltered. Although it cannot be excluded that enzymes other than desaturases were inactivated in *C. cohnii* by norflurazon and propyl gallate, our finding that the lipid content of the cells strongly decreased with both desaturase inhibitors does indicate that in *C. cohnii*, desaturases are involved. Consequently, the highly conserved fatty acid pattern of the lipids suggests that in *C. cohnii* the fatty acid synthesis is tightly controlled.

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References

Aursand M, Rainuzzo JR, Grasdalen H (1993) Quantitative high-resolution ^{13}C and ^{1}H nuclear magnetic resonance of omega-3 fatty acids from white muscle of Atlantic salmon (Salmo salar). J Am Oil Chem Soc 70:971-981

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long-chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6:123-129

Beach DH, Holz GG (1973) Environmental influences on the docosahexaenoate content of the triacylglycerols and phosphatidylcholine of a heterotrophic, marine dinoflagellate, *Crypthecodinium cohnii*. Biochim Biophys Acta 316:56

Beach DH, Harrington GW, Gellerman JL, Schlenk H, Holz GG (1974) Biosynthesis of oleic acid and docosahexaenoic acid by a heterotrophic marine dinoflagellate *Crypthecodinium cohnii*. Biochim. Biophys Acta 369:16-24

Bell MV, Henderson RJ (1990) Molecular species composition of phosphatidylcholine from *Crypthecodinium cohnii* in relation to growth temperature. Lipids 25:115-118

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911

Butte W (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxyde for transesterification. J Chromatogr 261:142-145

Christensen MS, Høy CE (1996) Effects of dietary triacylglycerol structure on triacylglycerols of resultant chylomicrons from fish oil- and seal oil-fed rats. Lipids 31:341-344

Cohen Z, Heimer YM (1990) Δ6 Desaturase inhibition, a novel mode of action of norflurazone. Plant Physiol 93:347-349

Harrington GW, Holz GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochim Biophys Acta 164:137-139

Henderson RJ, Mackinlay EE (1991) Polyunsaturated fatty acid metabolism in the marine dinoflagellate *Crypthecodinium cohnii*. Phytochemistry 30:1781-1787

Hornstra G (2000) Essential fatty acids in mothers and their neonates. Am J Clin Nutr 71:1262-1269

Kawashima H, Akimoto K, Shirasaka N, Shimizu S (1996) Inhibitory effects of alkyl gallate and its derivatives on fatty acid desaturation. Biochim Biophys Acta 1299:34-38

Kyle DJ, Sicotte VJ, Singer JJ, Reeb SE (1992) Bioproduction of docosahexaenoic acid (DHA) by microalgae. In: Kyle DJ, Radledge C (eds), Industrial Applications of Single Cell Oils. American Oil Chemists' Society, Champaign, Illinois, pp. 287-300

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. Lipid Technol 8:107-110

Leray C, Raclot T, Groscolas R (1993) Positional distribution of n-3 fatty acids in triacylglycerols from rat adipose tissue during fish oil feeding. Lipids 28:279-284

McLafferty FW, Tureček F (1993) Interpretation of Mass Spectra, 4th ed., University Science Books, Sausalito, USA

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293:290-293

Nettleton JA (1993) Are n-3 fatty acids essential nutrients for fetal and infant development? J Am Dietetic Assoc 93:58-64

Ratledge C (2001) Microorganisms as sources of polyunsaturated fatty acids. In: Gunstone FD (Ed.), Structured and Modified Lipids, Marcel Dekker, New York, pp. 351-399

Ratledge C, Kanagachandran K, Anderson AJ, Grantham DJ, Stephenson JM (2001) Production of docosahexaenoic by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. Lipids 36:1241-1246

Servel MO, Claire C, Derrien A, Coiffard L, de Roeck-Holzhauer Y (1994) Fatty acid composition of some microalgae. Phytochemistry 36:691-693

Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol 49:611-641

Sonnenborn U, Kunay WH (1982) Purification and properties of the fatty acid synthetase complex from the marine dinoflagellate, *Crypthecodinium cohnii*. Biochim Biophys Acta 712:523-534

Swaaf ME de, Rijk TC de, Eggink G, Sijtsma L (1999) Optimisation of docosahexaenoic acid production in batch cultivations by *Crypthecodinium cohnii*. J Biotechnol 70:185-192

Swaaf ME de, Sijtsma L, Pronk JT (2003) High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*. Biotechnol Bioeng 81:666-672

Timm A, Steinbüchel A (1990) Formation of polyesters consisting of medium chain length 3-hydroxy alkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl Environ Microbiol 56:3360-3367

Tocher DR, Leaver MJ, Hodgson PA (1998) Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. Prog Lipid Res 37:73-117

Viso AC, Marty JC (1993) Fatty acids from 28 marine microalgae. Phytochemistry 34:1521-1533

Wood BJB (1974) Fatty acids and saponifiable lipids. In: Steward, W.D.P. (Ed.), Algal Physiology and Biochemistry. Blackwell Scientific Publications, Oxford, pp. 236-65

Summary

This thesis focuses on the production of docosahexaenoic acid (DHA; 22:6), an ω-3 polyunsaturated fatty acid with applications in foods and pharmaceuticals, by *Crypthecodinium cohnii*. This chloroplastless heterotrophic marine microalga has been studied since the end of the nineteenth century and has been identified as a good producer of DHA. *C. cohnii* can accumulate lipid to over 20% of its biomass dry weight, with a high content of DHA (over 30% of the total lipid). Other polyunsaturated fatty acids represent less than 1% of the *C. cohnii*-derived oil.

The aim of the research described in this thesis was to identify relevant process parameters for the large-scale production of docosahexaenoic acid (DHA) with *C. cohnii.* Several cultivation protocols for *C. cohnii* were developed, analysed and optimised with respect to the production of biomass, lipid and DHA and solutions were sought for newly identified bottlenecks for industrial DHA production.

Chapter 2 describes batch cultivations of *C. cohnii* on media containing glucose, yeast extract and sea salt. Increasing amounts of yeast extract stimulated growth but influenced lipid accumulation negatively. Sea salt concentrations above half the average seawater salinity were required for good growth and lipid accumulation. *C. cohnii* was able to grow on a glucose concentration as high as 84 g l^{-1}, although concentrations above 25 g l^{-1} decreased the specific growth rate. Comparison of growth at 27 ^{o}C and 30 ^{o}C showed that the higher incubation temperature was more favourable for growth. However, lipid accumulation was higher at the lower incubation temperature. In a bioreactor the biomass concentration increased from 1.5 to 27.7 g l^{-1} in 74 h. In the final 41 h of the process the lipid content of the biomass increased from 7.5% to 13.5%. In this period, the percentage of docosahexaenoic acid of the lipid increased from 36.5% to 43.6%. The total amounts of lipid and docosahexaenoic acid after 91 h were 3.7 and 1.6 g l^{-1}, respectively.

In Chapter 3, so far unreported extracellular polysaccharides are described. The polysaccharides were produced by *C. cohnii* during batch growth on glucose, sea salt and yeast extract for 5 days. The polysaccharides caused an increased viscosity and a strong drop in the maximum oxygen transfer rate. The viscosity increased most markedly as cells entered the stationary phase. The polysaccharides varied in size (from 6 kDa to >1,660 kDa) and monomer distribution. A high molecular mass fraction (from 100 kDa to >1,660 kDa) and a medium

molecular mass fraction (6-48 kDa) were prepared. The high molecular mass fraction contained (on a molar basis) 71.7% glucose, 13.1% galactose and 3.8% mannose, whereas the medium molecular mass fraction contained 37.7% glucose, 19.8% galactose and 28.1% mannose. Other monomers present in both fractions were fucose, uronic acid and xylose. Monomers were coupled mainly via α-(1-3) links. Increased viscosity due to polysaccharide production complicates the development of commercial, high cell-density processes for the production of docosahexaenoic acid.

In Chapter 4, fed-batch cultivation was studied as an alternative fermentation strategy for DHA production by *C. cohnii*. Glucose and acetic acid were compared as carbon sources. For both substrates, the feed rate was adapted to the maximum specific consumption rate of *C. cohnii.* In glucose-grown cultures, this was done by maintaining a significant glucose concentration (between 5-20 g l^{-1}) throughout fermentation. In acetic acid-grown cultures, the medium feed was automatically controlled via the culture pH. A feed consisting of acetic acid (50% w/w) resulted in a higher overall volumetric productivity of DHA (r_{DHA}) than a feed consisting of 50% (w/v) glucose (38 and 14 mg l^{-1} h^{-1}, respectively). The r_{DHA} was even further increased to 48 mg l^{-1} h^{-1} using a feed consisting of pure acetic acid. The latter fermentation strategy resulted in final concentrations of 109 g l^{-1} dry biomass, 61 g l^{-1} lipid and 19 g l^{-1} DHA. These are the highest biomass, lipid and DHA concentrations reported to date for a heterotrophic alga. Vigorous mixing was required to sustain aerobic conditions during high-cell-density cultivation. This was complicated by culture viscosity, which resulted from the production of viscous extracellular polysaccharides (Chapter 3). These may present a problem for large-scale industrial production of DHA. Addition of a commercial polysaccharide-hydrolase preparation decreased the viscosity of the culture and the required stirring.

For commercial implementation of lab-scaled processes, scaling up is required. In Chapter 5, fed-batch cultivation of *C. cohnii* on acetic acid on pilot-plant scale (a 150-l bioreactor with 100-l working volume) and processing of the biomass are investigated. A procedure was developed to produce sufficient and viable cells to inoculate the 150-l bioreactor. *C. cohnii* was cultivated for 188 h in the 150-l bioreactor, yielding a final biomass concentration of 38 g dry weight l^{-1} with a DHA content of 12%. Centrifugation resulted in a 2.2 fold concentration of the dry matter in the heavy fraction. Subsequent spray-drying of the heavy fraction did not significantly affect the DHA content of the lipid.

In Chapter 6 the potential of ethanol as carbon source for DHA production by *C. cohnii* was studied. In shake-flask cultures, the alga was able to grow on media consisting of yeast extract, sea salt and ethanol. The specific growth rate was optimal with 5 g l^{-1} ethanol and did not occur at 0 g l^{-1} and above 15 g l^{-1}. In fed-batch cultivations with a controlled feed of pure ethanol, the cumulative ethanol addition could be much higher than 15 g l^{-1}, thus enabling a high final cell density and DHA production. The dissolved oxygen tension (DOT) was used to assess whether ethanol was limiting or inhibitory. The feed rate was increased when the DOT decreased after a small manual pulse of ethanol and was temporarily stopped when the DOT rose due to inhibition by high ethanol concentrations. In a representative fed-batch cultivation of *C. cohnii* with pure ethanol as the feed, 83 g l^{-1} dry biomass, 35 g l^{-1} total lipid and 12 g l^{-1} DHA were produced in 220 h. The overall volumetric productivity of DHA was 53 mg l^{-1} h^{-1}, which is the highest value so far reported for this alga.

In Chapter 7, the unknown pathway of DHA synthesis in *C. cohnii* is studied. Growth of *C. cohnii* on 1-^{13}C acetate and 1-^{13}C butyrate and subsequent analysis of the lipids with ^{13}C-NMR showed that only the odd carbon atoms of DHA were enriched. Apparently, two-carbon atom units were used as building blocks for DHA synthesis and butyrate was first split into two-carbon atom units prior to incorporation in DHA. A uniform distribution was found for the position of DHA in the lipids. When 1-^{13}C oleic acid was added to the growth medium, 1-^{13}C oleic acid was incorporated into the lipids of *C. cohnii* but was not used as a precursor for the synthesis of DHA. Desaturase inhibitors were able to inhibit lipid accumulation in *C. cohnii*. However, no change in the fatty acid profile was found. In contrast, in the arachidonic acid producing fungus *Mortierella alpina* these inhibitors both decreased the lipid content and altered the fatty acid profile. Our results can be explained by the existence of three tightly regulated separate systems in *C. cohnii*, namely for (1) the biosynthesis of saturated fatty acids, (2) the conversion of saturated fatty acids to monounsaturated fatty acids and (3) the *de novo* synthesis of DHA.

Samenvatting

Dit proefschrift richt zich op de productie van docosahexaeenzuur (DHA; 22:6), een ω-3 meervoudig onverzadigd vetzuur met toepassingen in voeding en farmaceutica, door *Crypthecodinium cohnii*. Deze heterotrofe marine microalg zonder chloroplasten wordt bestudeerd sinds het einde van de negentiende eeuw en staat bekend om zijn capaciteit om DHA te produceren. *C. cohnii* kan meer dan 20% van de droge biomassa aan lipiden ophopen met een hoog gehalte aan DHA (>30%). Andere meervoudig onverzadigde vetzuren komen niet of nauwelijks (<1%) voor in de lipiden van *C. cohnii*.

Het doel van het in dit proefschrift beschreven onderzoek was de identificatie van relevante procesparameters voor de grootschalige productie van DHA. Verschillende cultivatieprotocollen voor *C. cohnii* zijn ontwikkeld, geanalyseerd en geoptimaliseerd met betrekking tot de productie van biomassa, lipiden en DHA. Verder is gezocht naar oplossingen voor nieuw ontdekte beperkingen in industriële DHA productieprocessen met *C. cohnii*.

Hoofdstuk 2 beschrijft batchcultivatie van *C. cohnii* op media bestaande uit glucose, gistextract en zeezout. Hogere concentraties van gistextract stimuleerden weliswaar de groei maar de ophoping van lipiden werd negatief beïnvloed. Voor een goede groei en ophoping van lipiden waren zeezoutconcentraties boven ongeveer de helft van de gemiddelde zoutsterkte van zeewater vereist. *C. cohnii* bleek te kunnen groeien bij glucoseconcentraties van 84 g l^{-1} hoewel glucoseconcentraties boven de 25 g l^{-1} de groeisnelheid verlaagden. *C. cohnii* groeide sneller bij 30 ^{o}C dan bij 27 ^{o}C. De ophoping van lipiden was echter hoger bij 27 ^{o}C dan bij 30 ^{o}C. Tijdens batchcultivatie van *C. cohnii* in een bioreactor nam de biomassaconcentratie toe van 1.5 naar 27.7 g l^{-1} in 74 uur. In de laatste 41 uur van het proces steeg het lipidegehalte van de biomassa van 7.5 naar 13.5%. In deze periode ging het percentage DHA van de lipiden omhoog van 36.5 naar 43.6%. De totaal geproduceerde hoeveelheden lipiden en DHA waren na 91 uur kweken respectievelijk, 3.7 en 1.6 g l^{-1}.

In Hoofdstuk 3 wordt gerapporteerd over tot dusverre onbekende extracellulaire polysacchariden. Deze polysacchariden werden geproduceerd door *C. cohnii* tijdens batchgroei op glucose, zeezout en gistextract. Door de polysacchariden werd de viscositeit verhoogd en de maximale zuurstofoverdracht werd sterk verlaagd. De viscositeit nam vooral toe bij het bereiken van de stationaire fase. De polysacchariden varieerden zowel in grootte (van 6 kDa tot >1.660

kDa) als in monomeersamenstelling. Een fractie met zeer grote moleculen (van 100 kDa tot >1.660 kDa) en een fractie met relatief minder grote moleculen (van 6-48 kDa) werden van elkaar gescheiden en apart geanalyseerd. De fractie met de zeer grote moleculen bevatte (op een molaire basis) 71.7% glucose, 13.1% galactose en 3.8% mannose, terwijl de andere fractie bestond uit 37.7% glucose, 19.8% galactose en 28.1% mannose. In beide fracties kwamen ook de monomeren fucose, uronzuur en xylose voor. De monomeren waren vooral via α-(1-3) bindingen gekoppeld. De verhoging van de viscositeit door polysaccharideproductie bemoeilijkt de ontwikkeling van commerciële processen met hoge celdichtheden voor de productie van DHA.

In Hoofdstuk 4 werd fed-batch cultivatie bestudeerd als fermentatiestrategie voor DHA productie door *C. cohnii*. Glucose en azijnzuur werden onderling vergeleken als koolstofbronnen. De voedingssnelheid van beide substraten werd aangepast aan de maximale specifieke consumptiesnelheid van *C. cohnii*. Bij groei op glucose werd dit bewerkstelligd door de glucoseconcentratie tussen de 5 en 20 g l^{-1} te houden gedurende het proces. Bij groei op azijnzuur werd de voeding automatisch gecontroleerd via de pH van de cultuur. Een voeding van azijnzuur (50% w/w) resulteerde in een hogere gemiddelde volumetrische productiviteit van DHA (r_{DHA}) dan een voeding bestaande uit 50% (w/v) glucose (respectievelijk, 38 en 14 mg l^{-1} uur^{-1}). De r_{DHA} kon zelfs verder verhoogd worden tot 48 mg l^{-1} uur^{-1} door gebruik te maken van pure azijnzuur als voeding. Deze fermentatiestrategie resulteerde in concentraties van 109 g l^{-1} droge biomassa, 61 g l^{-1} lipiden en 19 g l^{-1} DHA na 400 uur kweken. In cultivaties van heterotrofe microalgen zijn dit tot dusverre de hoogst gerapporteerde waarden voor de biomassa-, lipide- en DHA-concentratie. Intensief mengen was noodzakelijk om aërobe condities te handhaven in de hoge-celdichtheids-cultivaties. Dit werd bemoeilijkt door de viscositeit van de cultures, veroorzaakt door de productie van visceuze polysacchariden. Dit kan een probleem opleveren voor de grootschalige productie van DHA. De toevoeging van een commercieel mengsel van polysaccharide hydrolases resulteerde in een lagere cultuurviscositeit waardoor met een lagere roersnelheid volstaan kon worden.

Opschaling is vereist om lab-schaal processen commercieel te kunnen implementeren. In Hoofdstuk 5 werd fed-batch cultivatie van *C. cohnii* op pilot-plant schaal (een 150-l bioreactor met een werkvolume van 100-l) en de verwerking van de biomassa bestudeerd. Een procedure werd ontwikkeld om voldoende en levensvatbare cellen te produceren om de 150-l bioreactor te enten. *C. cohnii* werd gedurende 188 uur gekweekt in de 150-l bioreactor, resulterend in een

biomassaconcentratie van 38 g drooggewicht l^{-1} met een DHA-gehalte van 12%. Centrifugatie leverde een 2.2-voudige verhoging van de biomassaconcentratie op in de zware fractie. Sproeidrogen van de zware fractie leidde niet tot een afname van het DHA-gehalte van de lipiden.

In Hoofdstuk 6 werd de mogelijkheid van ethanol als koolstofbron voor DHA-productie door *C. cohnii* bestudeerd. In schudkolfcultures was de alg in staat om op media bestaande uit gistextract, zeezout en ethanol te groeien. De specifieke groeisnelheid was optimaal bij 5 g l^{-1} ethanol en niet significant bij 0 en boven de 15 g l^{-1} ethanol. In fed-batch cultivaties met een gecontroleerde voeding van pure ethanol kan de cumulatieve ethanoladditie veel hoger zijn dan 15 g l^{-1} waardoor hoge einddrooggewichten en DHA-concentraties mogelijk worden. De opgeloste zuurstofspanning werd gebruikt om te bepalen of de ethanolconcentraties limiterend of vertragend waren. De ethanoltoevoer werd verhoogd wanneer de opgeloste zuurstofspanning omlaag ging bij een kleine handmatige puls ethanol en werd tijdelijk gestopt wanneer de opgeloste zuurstofspanning omhoog ging ten gevolge van hoge ethanolconcentraties. In een representatieve fed-batch cultivatie van *C. cohnii* met pure ethanol als voeding werden 83 g l^{-1} droge biomassa, 35 g l^{-1} lipiden en 12 g l^{-1} DHA geproduceerd in 220 uur. De r_{DHA} was 53 mg l^{-1} uur^{-1}; tot dusverre de hoogst gerapporteerde waarde voor deze alg.

In Hoofdstuk 7 werd de onbekende route van DHA-synthese in *C. cohnii* bestudeerd. Groei van *C. cohnii* op 1-^{13}C acetaat en 1-^{13}C butyraat en de daaropvolgende analyses van de lipiden met ^{13}C-NMR lieten zien dat alleen de oneven koolstofatomen van DHA verrijkt waren. Kennelijk worden eenheden van twee koolstofatomen gebruikt als bouwstenen voor DHA-synthese en wordt butyraat eerst gesplitst in eenheden van twee koolstofatomen alvorens ze worden geïncorporeerd in DHA. Een uniforme distributie van de positie van DHA in de lipiden werd gevonden. Toegevoegd 1-^{13}C oliezuur in het medium werd wel in de lipiden van *C. cohnii* ingebouwd maar niet gebruikt als precursor voor de synthese van DHA. Desaturase-remmers remden de lipideaccumulatie in *C. cohnii*. De vetzuursamenstelling van de lipiden werd echter niet veranderd. In de arachidonzuur-producerende schimmel *Mortierella alpina* verlaagden de remmers het lipidegehalte en veranderden tevens de vetzuursamenstelling van de lipiden. Onze resultaten kunnen worden verklaard met het bestaan van drie sterk gereguleerde afzonderlijke systemen in *C. cohnii* voor (1) de biosynthese van verzadigde vetzuren, (2) de omzetting van

verzadigde vetzuren naar enkelvoudig onverzadigde vetzuren en (3) de *de novo* synthese van DHA.

List of publications

Demel RA, Swaaf ME de, Hof R van 't, Mannock DA, McElhaney RE, Kruijff B de (1995) The specificity of glycolipid-preferredoxin interaction: requirements for membrane binding. Molecular Membrane Biology 12:255-261

Pilon M, Wienk H, Sips W, Swaaf ME de, Talboom I, Hof R van 't, de Korte-Kool G, Demel R, Weisbeek P, Kruijff B de (1995) Functional domains of the ferredoxin transit sequence involved in chloroplast import. Journal of Biological Chemistry 270:3882-3893

Flikweert MT, Swaaf ME de, Dijken JP van, Pronk JT (1999) Growth requirements of pyruvate-decarboxylase-negative *Saccharomyces cerevisiae* strains. FEMS Microbiology Letters 174, 73-79.

Sijtsma L, Springer J, Meesters PAEP, Swaaf ME de, Eggink G (1998) Recent advances in fatty acid synthesis in oleaginous yeasts and microalgae. Recent Research Developments in Microbiology 2:219-232

Swaaf ME de, Rijk TC de, Eggink G, Sijtsma L (1999) Optimisation of docosahexaenoic acid production in batch cultivations by *Crypthecodinium cohnii*. Journal of Biotechnology 70:185-192

Swaaf ME de, Grobben GJ, Eggink G, Rijk TC de, Meer P van der, Sijtsma L (2001) Characterisation of extracellular polysaccharides produced by *Crypthecodinium cohnii*. Applied Microbiology and Biotechnology 57:395-400

Swaaf ME de, Sijtsma L, Pronk JT (2003) High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*. Biotechnology and Bioengineering 81:666-672

Swaaf ME de, Pronk JT, Sijtsma L (2003) Fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii* on ethanol. Applied Microbiology and Biotechnology:in press

Swaaf ME de, Rijk TC de, Meer P van der, Eggink G, Sijtsma L (2003) Analysis of docosahexaenoic acid biosynthesis in *Crypthecodinium cohnii* by ^{13}C labelling and desaturase inhibitor experiments. Journal of Biotechnology:accepted for publication

Patent applications

Ratledge C, Anderson AJ, Kanagachandran K, Grantham DJ, Stephenson JC, Swaaf ME de, Sijtsma L (2001) Culture of *Crypthecodinium cohnii* for the synthesis of a polyunsaturated fatty acid. PCT International Application 2001 (Jan 18) WO 01/04338 A1; PCT/GB00/0295

Swaaf ME de, Pronk JT, Sijtsma L (2002) A process for production of polyunsaturated fatty acids by marine microorganisms. European patent application (May 1) number 02076752.1

Nawoord

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Martin

Curriculum vitae

Martin Egbert de Swaaf werd geboren op 4 april 1970 te Rotterdam. Hij behaalde in 1988 zijn Atheneum-B diploma aan het Theresialyceum te Tilburg en startte in datzelfde jaar een studie scheikunde aan de Universiteit Utrecht. Binnen deze studie volgde hij een keuzevak bij de vakgroep Enzymologie en Protein Engineering, een keuzevak bij de vakgroep Farmacochemie en een hoofdvak bij de vakgroep Biochemie van Membranen. Tijdens het hoofdvak verrichtte hij onder begeleiding van drs. R. van 't Hof (inmiddels gepromoveerd) en prof.dr. B. de Kruijff onderzoek naar de interacties tussen chloroplastlipiden en preferredoxine. Hij behaalde het doctoraal diploma in 1994. In 1995 begon hij een 2-jarige postdoctorale beroepsopleiding biotechnologie (Biotechnologie Opleidingen Delft Leiden, BODL) aan de TU Delft en behaalde het diploma in 1997. Binnen het stagejaar van deze opleiding deed hij 9 maanden onderzoek bij de vakgroep Microbiologie en Enzymologie van de TU Delft. Dit onderzoek richtte zich op het pyruvaatmetabolisme van de bakkersgist *Saccharomyces cerevisiae* en werd begeleid door dr. J.T. Pronk (momenteel hoogleraar). De laatste 3 maanden van het stagejaar bracht hij door bij het bedrijf Delta Biotechnology (Nottingham, Engeland). Hier bestudeerde hij de invloed van CO_2 op de productie van humaan serumalbumine door gisten onder begeleiding van dr. H. van Urk. Van 1997 tot 2002 verrichtte hij als wetenschappelijk medewerker van de afdeling Bioconversie van het Instituut voor Agrotechnologisch Onderzoek (ATO B.V.) te Wageningen onderzoek naar de productie van docosahexaeenzuur door de marine alg *Crypthecodinium cohnii*. Dit onderzoek ontwikkelde zich tevens tot promotieonderzoek met prof.dr. J.T. Pronk (Microbiologie en Enzymologie, TU Delft) als promotor en dr. L. Sijtsma (ATO B.V.) als begeleider. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Momenteel werkt hij bij Centocor B.V. te Leiden.