UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

Heterotrophic cultivation of microalgae as a source of docosahexaenoic acid for aquaculture

ENEKO GANUZA TABERNA
Grupo de Investigación en Acuicultura
Intituto Canario de Ciencias Marinas
Universidad de Las Palmas de Gran Canaria (IUSA)

Being a thesis submitted for the degree of

Doctor of Phylosophy

in the University of Las Palmas de Gran Canaria, 2008

Directors:
Prof. Marisol Izquierdo & Prof. Colin Ratledge

El haber nacido cerca del mar me gusta, me ha parecido siempre como un augurio de libertad y cambio...

Pio Baroja

Contents

List of Tables

List of Figures

List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AMP	Adenosine monophosphate
ARA	Arachidonic acid (20:4 n-6)
ATP	Adenosine triphosphate
ATTC	American type culture collection
CCMP	Culture collection marine phytoplankton
CDW	Cell dry weight
D	Dilution rate
DGLA	Dihomogammalinolenic acid (20:3 n-6)
DHA	Docosahexaenoic acid (22:6 n-3)
DPA-3	Docosapentaenoic acid (22:5 n-3)
DPA-6	Docosapentaenoic acid (22:5 n-6)
EDTA	Ethylenediamine tetraacetic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid (20:5 n-3)
FA	Fatty acid
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
GC	Gas cromatography
GLA	γ-linolenic acid (18:3 n-6)
GRAS	Generally recognized as safe
HUFA	Highly unsaturated fatty acids
LA	Linoleic acid
LNA	α-linolenic acid (18:3 n-3)
MOPS	3-(N-morpholino)propanesulfonic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OA	Oleic acid (19:1 n-9)
OCFA	Odd chain fatty acids
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PKS	Polyketide synthase
PUFA	Polyunsaturated fatty acids

r_{CDW}	Biomass volumetric productivity ($g\ l^{-1}\ h^{-1}$)
r_{DHA}	DHA volumetric productivity (mg DHA $l^{-1}\ h^{-1}$)
r_{FA}	Fatty acid volumetric productivity (mg FA $l^{-1}\ h^{-1}$)
rpm	Revolutions per minute
SCO	Single cell oils
SEM	Standard error of the mean
TFA	Total fatty acids
TRIS	Trishydroxymethylaminomethane
μ_{DHA}	Specific rate of DHA formation (mg DHA g $\mathrm{CDW}^{-1}\ h^{-1}$)
μ_{max}	Maximum specific growth rate

Acknowledgements

Acknowledgements

The following PHD thesis was made under the financial support from **Tomás de Zarate** scholarship from **Cabildo de Gran Canaria**. I would like to thank my supervisor, **Prof Marisol Izquierdo**, for making this possible, for her scientific, moral and professional support at all times. I am also very thankful my co-supervisor, **Prof Colin Ratledge**, for his scientific excellence, his comments and expertise taught to me throughout these years. To **Dr Alistair Anderson** I am especially grateful for initiating me on the scientific methodology. I am most grateful for the Britishness, along with many other valuable contributions made to the present thesis by my very friend **Elisa**. I am grateful to the **Instituto Canario de Ciencias Marinas**, **Universidad de Las Palmas de Gran Canaria** and **The University of Hull** for hosting me and nesting this research. Also, I would like to thank the staff of the **Lipid Research Centre** and **Grupo de Investigación en Acuicultura** for their collaboration. In particular, **Rachid** and **Orestes** showed me how to calm down and channel my scientific restlessness. **Eduardo** opened in me a new interest in IT, including Latex and Mac refinement. **Joaquin** has made the elegant covers I was missing. **Simon** showed me the art of fermentation. I would like to thank **Steven** for the English corrections. **Juan** showed me how to write back in Spanish. **Gorka** helped me with the bureaucratic matters. Ms. **Watford**, **Carmen**, **Debbie** and **Val** showed me how to hold the pipete. **Nivedita**, **Cath**, **Veena**, **Isabel** and **The Neibor** showed me to wisely wait for my results. **Ms Kennedy** helped me with the elementary analysis. **Tibi** and **Eyad** have been close to my fish larvae. **Elena**, **Lorena**, **Tache**, **Luis**, **Noe**, **Chano**, **Javi**, **Egoi**, **Maite**, **Raphaele** helped me by listening to the presentation of my results. I would like to thank different anonymous reviewers for opening a debate on the subject. I would also like to thank **Geert**, **Guido** and **Guillermo** and for the time they gave me to finish the dissertation. I would like to thank **Lolke**, **Juan Luis**, **Arnaud**, **Hector**, **Ricardo** for their comments to this thesis. Finally, my family showed me the Vito's way and overall I am grateful to this place, the **Canaries**.

Chapter 1

General introduction

1.1. Lipids and their role in living organisms

Lipids are a group of organic compounds that are insoluble in water, but soluble in organic solvents. Although there is not a structural definition, many lipids, namely those saponifiable, contain fatty acids, hydrocarbon chains with a carboxylic group at one end and a terminal methyl group at the other (n or ω carbon). The acyl chain may be saturated or unsaturated. The fatty acids commonly found in biological tissues possess an even-numbered carbon chain of between 12 and 24 atoms and 0 to 6 methylene-interrupted double bonds or unsaturations.

Being a heterogeneous and wide group of compounds, different classifications of lipids are currently used. For instance, according to their degree of polarity, they are known as polar or neutral lipids. The latter include fatty acids and their derivative glycerolipids, sterols, waxes and tocopherols, among others. Polar lipids include glycerophospholipids (currently named as phospholipids), glycoglycerolipids, sphingomyelin and glycosphingolipids.

Polyunsaturated fatty acids (PUFA) are fatty acids containing 18 to 24 carbon atoms with two or more unsaturations. The PUFA most frequently found in nature are: docosahexaenoic acid (DHA; 22:6 n-3) (Figure 1.1), docosapentaenoic acid (DPA; 22:5 n-3 or n-6), eicosapentaenoic acid (EPA; 20:5 n-3), arachidonic acid (ARA; 20:4 n-6), γ-linolenic acid (GLA; 18:3 n-6), α-linolenic acid (LNA; 18:3 n-3) and linoleic acid (LA; 18:2 n-6). Highly unsaturated fatty acids (HUFA), such as DHA, DPA, EPA and ARA, are those PUFA of 20 and 22 carbon atoms in their aliphatic chain with 3 or more unsaturations.

Fatty acids, as cell membrane constituents, are present in all life forms. Since cell membranes are responsible for receiving information and anticipating a response to any stress that perturbs the intracellular organization, changes in their fatty acid composition will directly affect cell function. Thus, the role of a fatty acid is not just structural or energetic, but also functional. Fatty acids are involved in several processes such as the control of membrane permeability, membrane plasticity, cell division, hormone formation

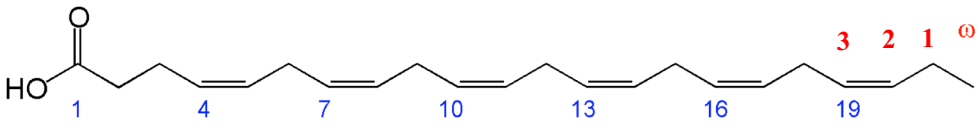


Figure 1.1: Schematic representation of Δ4, Δ7, Δ10, Δ13, Δ16, Δ19-docosahexaenoic acid (DHA; 22:6 n-3). The ω or n carbon is represented on the top and Δ carbon below the molecule.

and immune response.

Membrane fluidity is strongly determined by both environmental conditions and internal composition. Environment may change membrane fluidity, which acts as a sensitive receptor initiating cellular regulation (Beney and Gervais, 2001). Membrane fluidity is also affected by the content of unsaturated fatty acids in constituent phospholipids, which, due to their three-dimensional structure and lower hydrogen-hydrogen interactions, increase protein insertion. For example, the unsaturation index of membrane phospholipids of *Escherichia coli* decreased in response to low temperatures in order to maintain optimal viscosity (Nakayama *et al.*, 1980). In fact, PUFA improve stress tolerance and survival from heat and ethanol (Peyou-Ndi *et al.*, 2000), saline osmotic adaptation (Khaware *et al.*, 1995) or freezing (Giraud *et al.*, 2000). In particular, DHA possesses a very resistant structure to temperature and pressure changes in the membrane (Rabinovich and Ripatti, 1991) and modulates acyl chain structure and fluidity, phase behavior, elastic compressibility, permeability, flip-flop and protein activity (Stillwell and Wassall, 2003). In higher organisms, membrane DHA facilitates the fast conformational changes in membrane proteins associated with the visual and neuromuscular processes (Brown, 1994).

In addition to the role of membrane phospholipids in providing an environment for receptors, ion channels and enzymes, they are also a reservoir for lipid messengers. PUFA operate as precursors of potent bioactive mediators involved in cellular behavior and cell-to-cell communication (Kingsella *et al.*, 1990). These signaling molecules have great impact on the regulation of immune response (Calder *et al.*, 2002), blood clotting, inflammatory responses and homeostasis of the cell (Calder, 2002) and also in synaptic transmission and plasticity, neuroprotection and sleep/wake regulation (Chen and Bazan, 2005). The dihomogammalinolenic acid (DGLA; 20:3 n-6), ARA and EPA are liberated from membrane phospholipids by the action of phospholipase

A2 (Kingsella *et al.*, 1990). As free fatty acids, they compete for the same active site in both cyclo-oxygenase and lipoxygenase enzymes to produce the eicosanoid hormones, which includes prostaglandins, thromboxanes and leukotrienes, among other biologically active products. In the same way, DHA competes for these enzymes for the production of resolvins and protectines that are also involved in inflammatory control (Hong *et al.*, 2003; Serhan *et al.*, 2002). As a result, the fatty acid profile of the membrane phospholipids modulates the type of metabolic response of the organism. Dietary fatty acid deficiency or an imbalance in n-3/n-6 fatty acid ratio may produce pathological states connected with the immune suppression or inflammation such as: autoimmune disorders, rheumatoid arthritis, Crohn's disease, cancers of the breast, colon, and prostate, coronary heart disease and stroke, retinal and brain underdevelopment in infancy (Connor, 2000), as well as neurological disorders like epilepsy and Alzheimer's disease (Chen and Bazan, 2005).

Also, neutral lipids are the most efficient biological storage form of carbon and energy. They are hydrophobic molecules that enable the formation of aggregates in water providing a compact form of stored energy, readily used by the cell during starvation. Hence, in many organisms such as plant seeds, oleaginous microorganisms or certain fish, among others, glycerolipids are stored as an energy source before reproduction or adverse environmental conditions. Saturated and unsaturated fatty acids present in glycerolipids, containing up to 20-carbon chain are readily β-oxidized at the mitochondria to produce metabolic energy. In contrast, DHA is a poor mitochondrial substrate and requires peroxisomal β-oxidation (Madsen *et al.*, 1999).

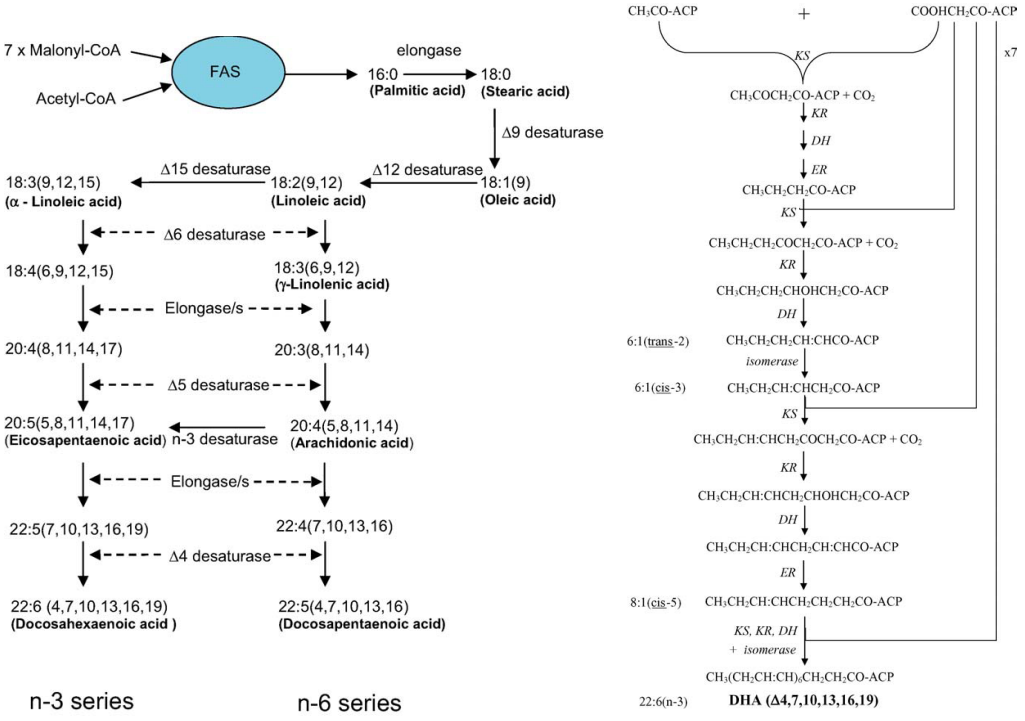
1.2. Lipid biochemistry

"*De novo*" fatty acid synthesis is anabolised by the fatty acid synthetase (FAS) enzyme-complex, responsible for acyl chain elongation. This process is initiated with the ATP-dependent condensation between malonate and acetyl co-A by 3-ketoacyl synthase, which is the rate limiting step. The enzyme 3-ketoacyl reductase together with NADPH introduces the hydroxyl radical at C_3 position. The dehydration between C_1 and C_3 by 3-hydroxyacyl dehydratase will produce the enoyl substrate used for the subsequent reduction by an enoyl reductase. As a result, the initial acyl chain is elongated by two carbon atoms. Thus, palmitic acid is the sequence product of seven elongation cycles that uses acetyl-CoA as a building block. This is a compulsory step in the formation of all the fatty acids in most eukaryotic organisms. Further synthesis of PUFA requires a cascade of reactions catalyzed by an alternating series of desaturase and elongase enzymes (Figure 5.1(a)). This is

the "longe-stablished" pathway for the synthesis of PUFA. Linoleic acid (LA; 18:2 n-6), α-linolenic acid (ALA; 18:3 n-3) and oleic acid (18:1 n-9) are the original precursors of, respectively, n-6, n-3 and n-9 series of PUFA. They all compete as substrates for Δ^6-desaturase , the first enzyme of the pathway. This is called a "front-end" desaturase because it introduces the unsaturation next to the carboxylic end (Δ^6 indicates a distance of six C-atoms from the α or carboxylic carbon). The following elongases and desaturases also operate next to the carboxylic end of the acyl chain, and, as a result, the position of the first double bound on the side of methyl terminus is conserved through the major series of fatty acids (n-3, n-6 and n-9). Generally, desaturases have a higher affinity for the correspondent n-6 substrate followed by n-3 and, finally, n-9 fatty acids. Thus, LA could interfere with further conversion of ALA and therefore the dietary n-3/n-6 ratio should be maintained at appropriate levels. Fatty acid desaturases are O_2-dependent enzymes, most of them membrane associated (Klein and Volkmann, 1975; Meyer and Bolch, 1963). Accordingly, this pathway is known as the "aerobic pathway" or "desaturase/elongase system", which is responsible for the production of the most common fatty acids present in nature.

As suggested above, the logic of elongase/desaturase chain of reactions indicates that DHA is the product finally derived from Δ^4- desaturase (Qiu *et al.*, 2001). However, unexpectedly, this enzyme has not yet been isolated from mammals. Alternatively, evidence indicates that DHA is synthesized through an extension of the traditional pathway called "Sprecher pathway" that avoids Δ^4-desaturation (Sprecher, 2002; Voss *et al.*, 1991). Two subsequent elongations of EPA followed by the action of Δ^6-desaturase produce 24:6 n-3. Eventually, 24:6 n-3 will complete one cycle of peroxisomal β-oxidation in order to produce DHA, 22:6 n-3. Thus, the pathway requires the migration of 24:6 n-3 from its usual location in the endoplasmic reticulum, into the peroxisome. Correspondingly, the n-6 pathway extension will produce DPA 22:5 n-6 from 22:4 n-6, again, without the involvement of Δ^4-desaturase.

As a novelty to the above mentioned, an alternative pathway for the synthesis of DHA that may be present in some marine bacteria (*Shewanella spp.* and *Moritella marina*), and at least one eukaryote (*Schizochytrium* sp.), has recently been proposed (Metz *et al.*, 2001). The genetic configuration of the fatty acid biosynthetic apparatus in those organisms suggests that DHA is synthesized by a novel bacteria-like polyketide synthase complex (PKS). The main difference with the pathway described above is that PKS does not require O_2 for desaturation (Figure 5.1(b)). This pathway is, to a certain extent, similar to the conventional FAS system, however, the elongating acyl-chain is not reduced after dehydration and, consequently, unsaturations



(a) Traditional FAS/desaturase/elongase pathway (b) Alternative PKS pathway

Figure 1.2: Pathways for the formation of PUFA in microorganisms using the 'conventional' FAS, desaturase/elongase pathway and alternative polyketide synthase complex (PKS) from Ratledge (2004). Although *Mortierella alpina* possesses a n-3 desaturase capable of converting a n-6 into n-3 fatty acid, in the majority of organisms the n-3 and n-6 series fall in two separate pathways. In the alternative PKS route, the desaturation is conserved during the addition of malonyl-Co rather than induced through a desaturase enzyme. As a result, this is an O_2-independent route with much more simple enzymatic and genetic structure. Enzymes: 3-ketoacyl synthase (KS); 3-ketoacyl-ACP reductase (KR);Dehydrase/isomerase (DH); Enoyl reductase (ER) .

are conserved. Instead, a *trans-cis* isomerization is performed by an isomerase enzyme. The "conventional" pathway requires an energetic surplus of two NADPH for the reduction of the naturally occurring double bond and posterior desaturation by fatty acid desaturase. Thus, PKS introduces the unsaturation in the acyl chain with a higher energetic efficiency than the conventional pathway (Ratledge, 2004).

From an ecological point of view, *de novo* synthesis of PUFA, is confined to certain ecological niches, mainly aquatic environments. Terrestrial vegetables are good sources of 18-carbon essential fatty acids, but vertebrates, including most marine fishes possess a limited capacity to convert these fatty acids to longer HUFA (Brenna, 2002). With few exceptions, the primary producers that are able to synthesize *de novo* HUFA are restricted to the aquatic environment. Due to the biological importance of these fatty acids, the trophic interactions are closely related to their presence (Masuda, 2003). For instance, mayor evolutionary episodes of *Homo sapiens* have been connected to the aquatic environments through dietary lipids, apparently, due to the vital role of HUFA in neural development (Broadhurst and Wang, 2002; Crawford *et al.*, 1999). Moreover, HUFA predict the carbon transfer between primary producers and consumers in freshwater ecosystems (Müller-Navarra *et al.*, 2004, 2000). In nature, the food chain will ultimately provide fish with the majority of essential HUFA through diet (Ackman *et al.*, 1964). In particular, marine fish and crustacean used in aquaculture do not synthesise most of HUFA and consequently they rely upon dietary lipids coming from fish processing industry (wild catch). Indeed, it is not yet possible to harvest the ocean for the primary producers, ultimately responsible for the production of this nutrient, thus the ecological impact of aquaculture feed requirements is mainly upon fisheries.

1.3. Importance of HUFA for marine finfish

The first attempts to culture marine fishes with rotifers reported high larval mortalities when HUFA were absent from the diet (Watanabe *et al.*, 1983). In fish larvae, essential fatty acid requirements are more evident because during fast-growing stages, the body cannot synthesise all the necessary components at the required rate. Thus, as soon as the larva depletes the nutrients from the yolk sack, HUFA deficiencies are quickly manifested in terms of high mortality, poor growth and reduced activity (Izquierdo, 1996, 2005). It is generally understood that eggs contribute with all the necessary components for the embryo and larval development before external feeding begins. In view of the resistance to dietary modification of the egg lipid-composition relative to other tissues, it was inferred that this may reflect

the requirements for the following life stages of the species. In fact, the eggs of marine fishes (Sargent *et al.*, 2003), including seabream (Mourente and Odriozola, 1990), contain high quantities of HUFA and phospholipids. Moreover, seabream hatchlings under starvation tend to conserve the valuable n-3 HUFA of membrane phospholipids preferentially to other fatty acids of the body (Koven *et al.*, 1989). The first successful attempts to culture marine fish larvae utilised n-3 HUFA from marine oils (emulsified with lecithin) as enrichment for live prey (Watanabe *et al.*, 1983).

DHA, EPA and ARA are essential fatty acids for the normal growth, development (Watanabe, 1982) and reproduction (Izquierdo *et al.*, 2001) of marine fish. Unlike freshwater fish, the marine fish so far studied possess a limited, almost negligible, capacity to synthesise LNA to EPA and LA to ARA (Owen *et al.*, 1975; Tocher *et al.*, 2003). In seabream, the conversion rate is mainly limited due to the low activity of Δ^5-desaturase (Tocher and Ghioni, 1999). Thus, HUFA are obtained by fish through the diet and incorporated selectively in the body tissues. However, not only optimum levels of HUFA must be provided, but also the different fatty acids at the required ratios, which are species-specific, as well as, dependent on the life stage and metabolic conditions (i.e. smoltification) (Sargent and Tacon, 1999).

Marine fish larvae appear to possess higher specific requirements for DHA than juveniles and pre-adults. This fatty acid is incorporated in the developing visual and neural tissues (Mourente, 2003), which, at this stage, account for a higher proportion of body weight relatively to other fully-developed fish. DHA deficiency in the diet is related to impaired ability of herring larvae to capture prey at natural light intensities (Bell *et al.*, 1995), retarded development of normal behaviour of yellowtail (Masuda *et al.*, 1998) and gilthead seabream (Benítez-Santana *et al.*, 2006), increasing incidence of skeletal deformities (Cahu *et al.*, 2003), immunosuppression (Montero *et al.*, 2004), malpigmentation and impaired eye migration in flatfish (Bell *et al.*, 2003; Mc Evoy *et al.*, 1998). DHA and EPA are competitively incorporated into cell membranes; however, the former is superior as an essential fatty acid for growth performance and stress resistance in red sea bream among other species (Watanabe *et al.*, 1989; Watanabe and Kiron, 1994). HUFA requirements of seabream larvae are as low as 1.5 % of dry weight (Rodríguez *et al.*, 1998), but it increases four times this value with an increasing preponderance of EPA over DHA (Rodríguez *et al.*, 1994b). The optimum DHA:EPA ratio is about 10:5 (Rodríguez *et al.*, 1994a, 1997) and it decreases to less than 10:10 in seabream juveniles (Ibeas *et al.*, 1997). Because ARA is less abundant in fish tissues than the other essential fatty acids it has received less attention. However, ARA is recognised as one of the primary eicosanoid precursors together with EPA (Ganga *et al.*, 2006). Dietary implementation

with ARA contributes to improve the stress response and better survival of seabream larvae (Bessonart *et al.*, 1999; Van Anholt *et al.*, 2004), but any improvements in growth by ARA appears to be masked by the influence of dietary DHA:EPA ratio (Ibeas *et al.*, 1997; Robin and Peron, 2004).

These three essential fatty acids have complex interactions that should be considered before defining the dietary requirements. The first level of competitive interactions between HUFA might occur at the digestive system. The bile salt-activated lipase (BAL) from red seabream (*Pargus major*) possesses higher affinity for EPA and ARA than for DHA when esterified to dietary neutral lipids (Iijima *et al.*, 1998) and might contribute to differential assimilation. Afterwards, during lipid absorption and transport, the addition of polar lipids in microdiets increases the incorporation of n-3 HUFA, preferentially DHA, in the polar lipids of seabream larvae (Salhi *et al.*, 1995). In fact, the acylases and transacylases that esterify fatty acids into the different phospholipids possess specific enzymatic affinities that eventually might interfere with the transport and incorporation to the different tissues. For instance, ARA is almost exclusively found attached to the second position of the glycerol from phosphatidylinositol (PI), while DHA is mainly attached to the second position of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Sargent *et al.*, 2003). Dietary DHA competes with EPA for the second position of PE, hence a high dietary DHA/EPA ratio increases DHA esterification to PE and improves growth in seabream (Izquierdo *et al.*, 2000). In addition to the selectivity of fatty acid assimilation, there is an incidence of fatty acid turnover on the final composition of tissues due to the metabolism of synthesis and catalysis of HUFA. The enzymes performing desaturation and elongation are activated by the presence of their substrate and inhibited by their product. As mentioned previously, the conversion rate of n-3 and n-6 PUFA to longer HUFA is poor due to the low activity of certain enzymes of the pathway, either Δ^5-desaturase or C_{18} to C_{20} fatty acid elongase (Tocher *et al.*, 2003). Thus the balance of n-6 and n-3 precursors, LA and ALA, will not initially alter the proportion of biological active forms of essential fatty acids (EFA). However, regarding the theory of the so-called "Sprecher shunt", that has been demonstrated at least in rainbow trout (Buzzi *et al.*, 1997), not only 18:2 n-6, 18:3 n-3 but also 24:4 n-6 and 24:5 n-3 are substrates for Δ^6-desaturation. It has not been demonstrated whether the same Δ^6-desaturase enzyme is performing in C_{18} and C_{24} fatty acids, but it suggests that a more complex competition occurs between the different substrates. On the other hand, fatty acid catabolism tends to preferentially conserve HUFA to other saturated fatty acids, as functional and structural components in membrane phospholipids. However, HUFA are also susceptible to selective β-oxidation that modifies the fatty acid profile of the body from the initial dietary profile.

Tuna lipids contain a high DHA to EPA ratio that might be a consequence of selective catabolism of dietary EPA relative to DHA (which is oxidized in the peroxisome) (Sargent *et al.*, 2003). Finally, another competition between HUFA occurs for the synthesis of eicosanoids. ARA and EPA are substrates of lipoxygenases for the production of different leukotrienes and of cyclooxygenases for the production of prostaglandins (Kingsella *et al.*, 1990). These signaling molecules, derived from both fatty acids, compete for the same cell receptors and might have antagonistic physiological activities against each other (Whelan, 1996). Although ARA is usually present in a lower proportion than EPA, it possesses a higher affinity for cyclooxygenase. In turn, EPA possesses higher affinity for lipoxygenase. The resulting physiological activity will be determined by the relative levels of both fatty acids in PI. Due to the complex interactions between HUFA and the possibility of competitive interference, the proportion in which the three essential fatty acids are present in diet must be defined in terms of ratio all together, as well as in absolute amounts (Izquierdo, 1996; Sargent and Tacon, 1999; Izquierdo *et al.*, 2000).

1.4. Importance of HUFA for human health

Linoleic (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3) are considered to be EFA in most terrestrial vertebrates. These fatty acids are precursors of a variety of very important HUFA, but cannot be synthesized *de novo* in humans. Their deficiency is very rare in the diets from industrialized countries because these fatty acids are present in most higher plants. However, HUFA synthesis through the series of elongation and desaturation in the tissues seems to occur at an insufficient rate at certain stages of life (Burdge and Wootton, 2002; Emken *et al.*, 1994; Gerster, 1998; James *et al.*, 2003; Nakamura and Nara, 2003). HUFA syntheses depend upon age, sex, dietary intake, disease presence and challenging metabolic processes. Therefore, dietary LA and ALA do not completely solve HUFA deficiency in diet and the lack of HUFA in the diet could cause a broad variety of long-term health problems (Bistrian, 2003). In contrast, dietary HUFA that can be partly retro-converted to lower chain fatty acids, will reverse the symptoms of LA and ALA deficiency (Hansen *et al.*, 1986; Sprecher *et al.*, 1995). Based on these findings, DHA, EPA and ARA are also considered essential fatty acids for maintaining healthy body functions (Cunnane, 2000).

Many diverse health benefits have been found to be related to n-3 PUFA intake (Simopoulos, 1999). There is considerable evidence indicating the positive effects of dietary n-3 PUFA in the prevention of cardiovascular diseases (Lichtenstein, 2003), atherosclerosis (Ristic and Ristic, 2003), hormone-

dependent cancers (Terry *et al.*, 2003), diabetes (Brenner, 2003) and autoimmune diseases, such as, rheumatoid arthritis (Cleland *et al.*, 2003; Simopoulos, 2002). In particular, DHA is involved in the protection of neurological tissues from oxidative stress (Yavin *et al.*, 2002), development of brain (Wainwright, 2002) and retina (Jeffrey *et al.*, 2001), prevention of mental bipolar disorder (Rapoport and Bosetti, 2002), attention-deficit hyperactivity (Burgess *et al.*, 2000), schizophrenia (Kemperman *et al.*, 2006), depression (Sontrop and Campbell, 2006) and Alzheimer's disease (Cole *et al.*, 2005).

Although dietary HUFA levels depend on the dietary habits of the different social groups and countries, the minimum recommended ingestion includes at least two oil-rich fish-based meals per week and, particularly, the inclusion of DHA and ARA in all infant formulas regardless of the intake of LA and ALA (FAO/WHO, 1994). The Dietary intake of n-6 LA from vegetable oils in western countries is far more abundant than n-3 ALA intake, which mainly comes from green leaf vegetables (Kris-Etherton *et al.*, 2000; Nettleton, 1991; Sanders, 2000). In the absence of a HUFA dietary source, the body's n-3/n-6 imbalance is conserved, if not enhanced, through desaturation and elongation to longer HUFA. Since both n-families are precursors of eicosanoids, their proportion (n-3/n-6) should be balanced through diet to ensure adequate functioning of metabolism. Given that n-6 intake is currently high in western societies, dietary n-3/n-6 balance must be corrected by the dietary intake of DHA and EPA of around 0.5 $g\ d^{-1}$ for infants, 1 $g\ d^{-1}$ for adults and 2–4 $g\ d^{-1}$ for patients with hyperglycemia or under high cardiovascular diseases risk (Kris-Etherton *et al.*, 2002). At present, fish, including those produced by aquaculture, constitute the main source of n-3 HUFA in diet.

1.5. Fish oil as a source of HUFA

Nowadays, vegetable oils, which are rich in n-6 fatty acids, account for the majority of the natural edible oils in the world market (O'Mara, 1998). Animal lipids, such as lard, are cheaper but they are not considered as healthy as vegetable oils due to the lack of PUFA and their high content of saturated fats. Fish is the main source of n-3 HUFA, in particular DHA and EPA (Nettleton, 1991). The traditional sources of HUFA for feedstock are fish oil and fishmeal derived mainly from small oily fishes such as anchovy, capelin and blue whiting that are bony and less suitable for human consumption.

Fish oil contains a complex and variable fatty acid profile, its quality depending on the fish species, season, fishing location and processing. The most important fatty acids in fish oil include DHA and EPA in a ratio of about 10:20. Soybean oil is probably the main alternative to fish oil in the

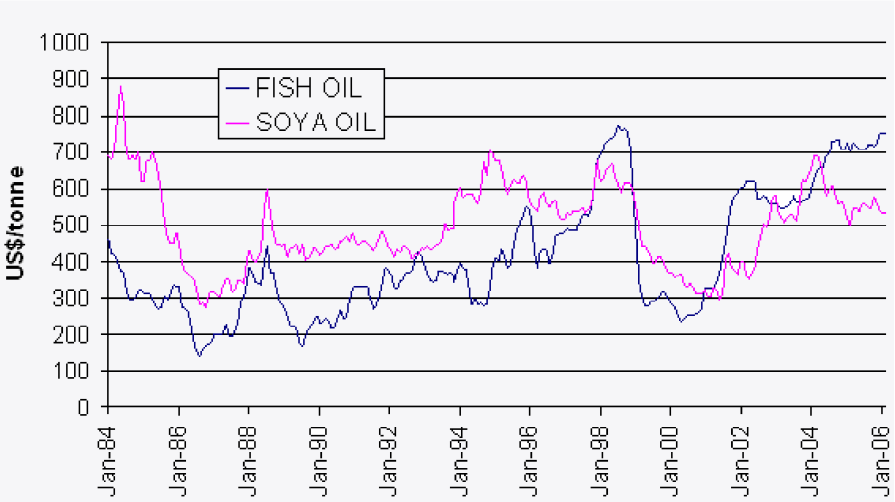


Figure 1.3: Fish oil and soybean oil price (Josupeit, 2006).

market, but, with other vegetable sources, it lacks HUFA. Hence, although soybean oil was 40 % cheaper than fish oil in 2006 (Josupeit, 2006) it is less desirable, from a nutritional point of view, than fish oil.

Peru is the main world producer of fish oil, followed by Scandinavian countries and Chile. Unfortunately, the fisheries of anchovy from the Peruvian coast are strongly influenced by climatic events. In fact, the world price of fish oil and fishmeal in 1998 increased to US $ 750 ton^{-1} due to a strong "El Niño" phenomenon influencing the fish captures (Figure 1.3). Otherwise, in common a climatic condition, the annual world production of fish oil remained stable (1.0–1.7 million ton) in the last two decades, but the price tends to gradually increase due to increasing demand. At the beginning of 2006 the fish oil price attained US $ 750 ton^{-1}. Thus, the historical maximum attained in 1998 due to "El Niño" was surpassed due to increasing demand for the product. Although the International Fishmeal and Fish Oil Organization (IFFO) is against a background of changing use due to market forces (Shepherd *et al.*, 2005), the fact is that whatever the demand is for fish oil and fishmeal, fishing quota are applied to most of the fisheries; thus catches are not expected to grow with demand.

Aquaculture, pork, poultry and ruminants are the main sectors demanding the global production of fish oil and fishmeal. Particularly, aquaculture is at present the fastest growing food sector, accounting for one third of the total fish consumed by men. Global aquaculture production is expected to increase at an annual rate of 4.5 % over the period 2010–2030 (Brugère and

Ridler, 2004). Hence, it is the main factor responsible for the increasing demand of fish oil and fishmeal (Naylor *et al.*, 2000; Tacon, 2004). So far, this demand, has been overcome through a shift from using the fish oil in livestock and margarine fabrication into using it for marine fish and crustacean feeds. Certainly, the key element inducing this reorganization of fish oil usage is the nutritional value of n-3 fatty acids. Nevertheless, in view of the mentioned predictions, it is clear that demand for fish oil from aquaculture will exceed supply in the very near future.

Much research has been done toward increasing growth of aquaculture in a sustainable way (Naylor *et al.*, 2000). Several approaches to prevent fish oil shortage are possible in order to protect aquaculture growth (Sargent *et al.*, 1999). In the first place, as mentioned above, the reorganization of fish oil usage, currently in process, is based on the requirements of omega-3 fatty acids. Hence, its use in terrestrial animals is being diverted to the aquaculture of marine species and particularly to starter diets since the fast-growing early stages possess higher requirements for HUFA. Secondly, as HUFA are the key nutrient in fish oil, raw material processing should be improved in order to optimize the yield in terms of HUFA. Thirdly, a step forward would be to include partial replacement of the dietary lipids sources with vegetable oil sources. The degree of substitution in aquafeeds that maintains fish growth, welfare and product quality to the consumer has been defined in some carnivorous fishes (Izquierdo *et al.*, 2003, 2005; Bell *et al.*, 2005; Ganga *et al.*, 2005). These implementations will help to lessen the shortage of fish oil through the enhancement of the conversion efficiency from pelagics to farmed fish and by improving the sustainability of aquaculture production. Nevertheless, in spite of all these actions directed to prevent a fish oil shortage, the definite solution to this problem relies on the production of alternative sources of HUFA.

1.6. Single cell oils as source of HUFA

Single cell oils (SCO), also known as microbial oils, are commercially extracted from unicellular organisms (Ratledge, 1992). Microbial oil, a relatively new concept in the biotechnology industry, is now gaining interest due to its capacity to produce HUFA at large scale. Although currently, the bulk source of HUFA, in terms of high quantity-low price is fish oil, there is big interest in SCO as a potential substitute, as it possesses several advantages over the traditional source. Fish oil refers to a complex blend of more than 40 different fatty acids the proportion of which varies with species and season, whereas microbial sources could provide, throughout the year, more concentrated sources of a particular fatty acid (Table 1.1). This fatty

Table 1.1: Major fatty acids of various oleaginous organisms considered for fatty acid production and their volumetric productivities (r_{FA}).

Organisms	14:0	16:0	16:1	18:0	18:1	18:2	18:3 n-6	20:4 n-6	20:5 n-3	22:5 n-6	22:6 n-3	r_{FA} (mg FA l^{-1} h^{-1})
Schizochytrium SR21[a]	13	29	12	1	1	1	-	-	-	12	36	145[b]
Schizochytrium ATCC20888[c]	-	-	-	-	-	-	-	-	-	-	25–40	≃417
Thraustochytrium aureum[d]	3	8	-	-	16	2	-	3	-	-	52	3
Ulkenia sp.[a]	3	30	-	1	-	1	-	-	-	11	46	-
Crypthecodinium cohnii[a]	20	18	2	-	15	0.4	-	-	-	-	39	53[e]
Nitzchia laevis[f]	23	33	-	-	33	2	1	1	4	-	-	10.4
Mortierella alpina 1S4[a]	0.2	6	-	2	4	4	2	70	-	-	-	54[g]
Mucor circinelloides[h]	22	-	1	6	40	11	18	-	-	-	-	4.8[i]

[a] (Ratledge, 2004), [b](Yaguchi *et al.*, 1997), [c](Barclay *et al.*, 2003), [d](Singh *et al.*, 1996), [e](de Swaaf *et al.*, 2003), [f](Barclay *et al.*, 1994; Kyle and Gladue, 1993), [g](Higashiyama *et al.*, 1998), [h](Ratledge and Wynn, 2002), [i](Kennedy *et al.*, 1993)

acid could be easily purified because it usually does not interfere with other PUFA. Certain fish oils, such as tuna oil, present predominant quantities of a particular fatty acid, DHA (Saito *et al.*, 1997), but this oil is scarce and expensive. Moreover, there is a risk with fish oils being vehicles of marine pollutants that might eventually accumulate in aquacultured organisms (Hites *et al.*, 2004). In contrast, oleaginous microbes are cultured under controlled conditions (bioreactors) providing an organic-food-grade product, free of contaminants. Finally, fish oil implementation in functional foods and nutraceuticals is constrained due to stability problems and the characteristic fishy taste and odor, whereas SCO might possess a higher oxidative stability due to the natural antioxidants synthesized within the cell (Sargent *et al.*, 1997) and lack that unpleasant taste and odor.

SCO are produced by oleaginous microorganisms, those able to produce lipid in quantities that justify a commercial consideration. Usually, this value is determined by definition to be higher than 20 % of cell dry weight (CDW) (Ratledge and Wynn, 2002), which will necessarily include a proportion of non-essential storage lipids. Those storage lipids are generally accumulated within the oleaginous cell as triacylglycerides, in lipid-droplets that are easily extracted as SCO. Only a few microorganisms (yeast, fungi and algae) are able to accumulate lipids between 20–70 % CDW (depending on microbial species and environmental conditions). The lipid accumulation process has been described for heterotrophic organisms, which are so far the only suc-

cessful commercial realities in the HUFA specific, SCO industry (Ratledge, 2002a).

Lipogenesis is usually associated with some nutrient limiting conditions other than the carbon source, particularly the nitrogen source (Gill *et al.*, 1977; Granger *et al.*, 1993; Ratledge and Wynn, 2002). Thus, lipid accumulation can be induced by manipulating the carbon-to-nitrogen ratio of the culture medium. In batch culture, this is usually a bi-phasic process. The first phase is characterized by rapid cell growth and is followed by the stressed phase, where the remaining carbon is allocated to the production of lipids. The biochemical events leading to lipid accumulation were extensively studied and recently reviewed by Ratledge and Wynn (2002); Ratledge (2004). Following nitrogen exhaustion in the culture medium, the little ammonia contained in the intracellular pool of AMP is scavenged through the action of AMP deaminase. As a result, isocitrate dehydrogenase (ICDH), which is AMP dependent (only in oleaginous microorganisms), is down-regulated resulting in citrate accumulating into mitochondria and being liberated to the cytosol (Figure 1.4). From a biochemical point of view, lipid accumulation requires the presence of ATP: citrate lyase (ACL) which is responsible for the cleavage of citrate in the cytosol and provides acetyl-coA as a building block for fatty acid synthesis (Ratledge and Wynn, 2002). This enzyme is crucial in most oleaginous organisms but is not the sole enzyme responsible for the lipid accumulation process. The malic enzyme is also essential to the process, converting malate to pyruvate and providing an internal pool of NADPH for lipid synthesis. Each building block introduced in the fatty acyl chain requires acetyl-coA and two NADPH to be reduced. Thus, both ATP:citrate lyase and malic enzyme are crucial in providing the key components for lipid synthesis.

1.7. DHA-producing microorganisms

Marine microorganisms, including bacteria, algae and fungi, appear to be the main producers of HUFA in the marine food chain. Considering the high microbial biodiversity of the oceans (Sogin *et al.*, 2006) and the large percentages of undescribed species within the group of marine microorganisms (Colwell, 1997), they constitute good screening sources for potential SCO producers. DHA is a high added-value product with potential for exploitation through biotechnology as it can not be more cheaply produced by terrestrial plants. Bacteria are probably not suitable for DHA production, firstly because they do not accumulate large amount of triacylglycerides, and secondly because they may contain unusual lipids that are not typically present in feeds and foods (Ratledge, 2002a). Fungi of the genus *Mortierella*

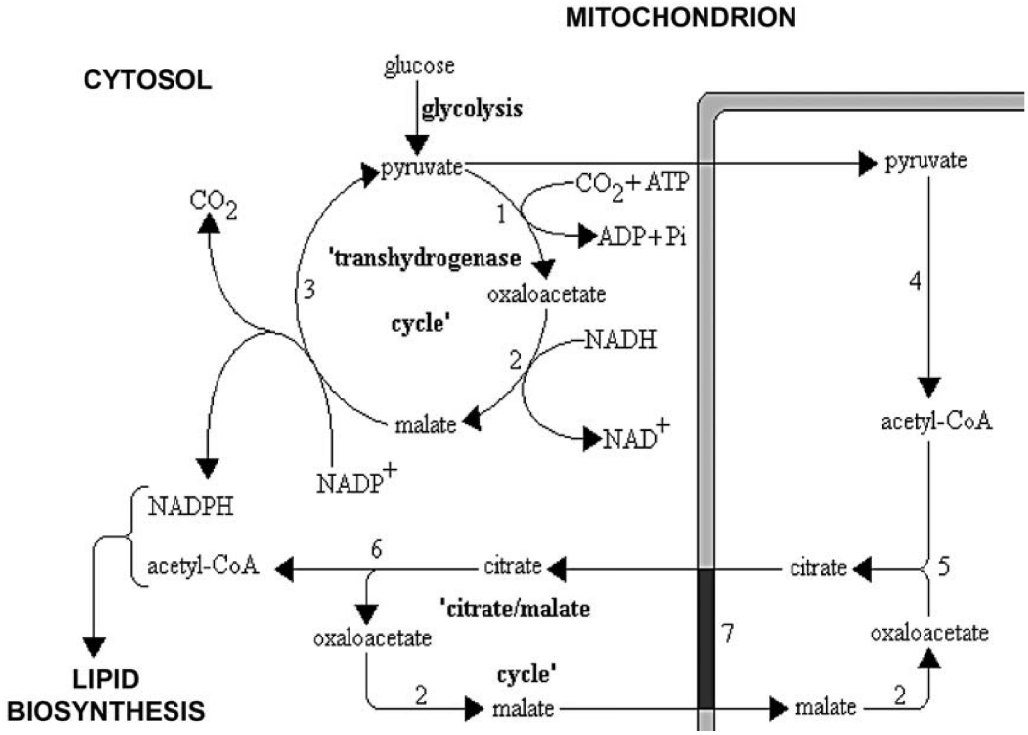
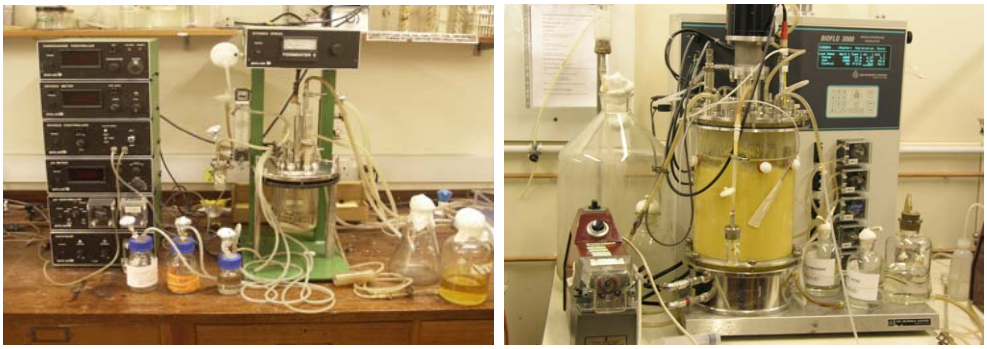


Figure 1.4: Biochemistry of lipid accumulation in oleaginous microorganisms (Ratledge (2004)). The citrate/malate cycle and the cytosolic 'transhydrogenase' cycle provide sufficient precursors of acetyl-CoA and NADPH for lipogenesis. Enzymes: 1-pyruvate decarboxylase, 2-malate dehydrogenase, 3-malic enzyme, 4-pyruvate dehydrogenase, 5-citrate synthase, 6-ATP:citrate lyase

are grown for the production of ARA, however, fungal based technologies may not be appropriate for DHA production due to the high fermentation times and consequent low productivities of the selected organisms (Barclay *et al.*, 1994). Marine algae, broadly used in aquaculture as DHA sources, have proved to be far more productive when they can be grown in heterotrophic rather than in phototrophic conditions (Apt and Behrens, 1999; Zaslavskaia *et al.*, 2001). The light-shading effect of the growing cells in phototrophic cultures limits the maximum cell densities attainable in those systems. By means of increasing the surface-to-volume ratio of the culture broth, light utilization and cell densities can be slightly increased, but in return that complicates the scaling-up feasibility. As a result, the overall cost of microalgae biomass produced in open ponds such as raceways (US \$ 15–20 kg^{-1}) or closed systems such as photobioreactors (> US \$ 50 kg^{-1}) (Borowitzka, 1997), is not competitive with the biomass cultivated in fermenters (< US \$

5 kg^{-1}) (Gladue and Maxey, 1994).

Heterotrophic microorganisms obtain the required metabolic energy from the oxidation of an organic substrate. They are grown in fermenters under sterile conditions (see Figure 1.5), in the dark if necessary, under a highly-controlled environment (temperature, dissolve O_2, airflow, stirring speed, pH control). The stability of culture conditions sustains rapid growth and lipid accumulation of the organism in a reproducible way. Under optimal growth conditions, provided with an adequate air supply, large-scale fermenters can produce over 100 g CDW l^{-1}. High cell densities greatly decrease the volume of broth that must be processed during the harvesting step which, otherwise, can contribute with one third of the production costs (Barclay *et al.*, 1987). The main factors influencing the cost of fermentative DHA production are cultivation scale of and overall DHA productivity of the system (mg DHA l^{-1} h^{-1}) (Sijtsma *et al.*, 1998). Overall DHA productivity depends on growth rate, as well as lipid and DHA content of the cell. Environmental and nutritional aspects can be implemented in order to tailor the lipid content and the fatty acid profile. For example, utilization of C_2 substrates, like acetate or ethanol, seems to improve lipid formation by means of supplying the cell with more direct precursors of acetyl-CoA (Sijtsma and de Swaaf, 2004). Low dissolved O_2 tension of the culture may decrease the degree unsaturation of some organisms due to the down regulation of the O_2-dependent desaturases. O_2 transfer related environmental factors, namely aeration and stirring speed, have been employed in order to tailor cocoa butter equivalents from *Mucor circinelloides* (Roux *et al.*, 1995). Currently, three commercial processes exist for the production of microbial DHA based in species belonging to thraustochytrids and dinoflagellates (Ratledge, 2004).



(a) 1 l Braun Biolab Fermenter. (b) 10 l New Brunswick BioFlo 2000.

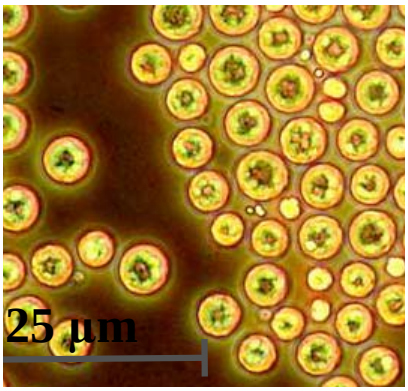
Figure 1.5: Fermentation facilities for single cell oil production.

Thraustochytrids are marine, obligate heterotrophs that develop ecto-

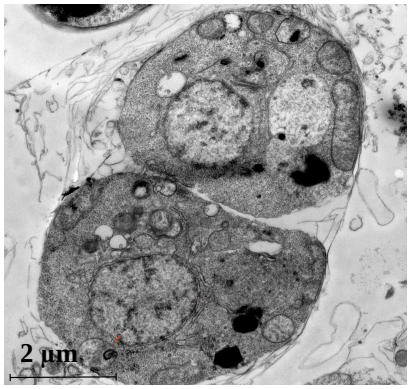
plasmic nets in a similar way as fungi. They were originally classified as phycomycetes (algae-like fungi) due to their similarities with fungi, but later they have been assigned to the kingdom Chromista (Cavalier-Smith *et al.*, 1994). According to their molecular characteristics, which are closely related to algae, they are assigned, together with labyrinthulids, to a new phylum of heterokont algae. *Schizochytrium* sp. (see Figure 1.6(a)–1.6(d)) is a thraustochytrid which can contain up to 78.6 % of CDW as fatty acids, with 33.3 % being DHA (Yaguchi *et al.*, 1997). The overall DHA volumetric productivity could be 150 $mg\ l^{-1}\ h^{-1}$, while other processes based on *Schizochytrium*-related organisms claim DHA productivities as high as 417 $mg\ l^{-1}\ h^{-1}$ (Barclay *et al.*, 2003). In addition to DHA, *Schizochytrium* sp. produces around 10 % TFA of docosapentaenoic acid (22:5 n-6; DPA), as well as odd-chain fatty acids, such as 15:0 and 17:0. OmegaTech Inc., Boulder, CO, now owned by Martek Biosciences Corp., is commercially producing this organism, although the process details remain proprietary at present. SCO from *Ulkenia sp.*, another thraustochytrid, possesses a similar fatty acid profile to *Schizochytrium* sp. and is commercially produced by Nutrinova GmbH, Frankfurt, Germany. Very little information is available about this microorganism.

Crypthecodinium cohnii, formerly known as *Gyrodinium cohnii*, is a chloroplastless marine organism that belongs to the order Dinophyceae which can be isolated from rotting seaweed (see Figure 1.6(e)–1.6(f)). This organism can produce up to 50 % of its dry weight as lipids with 32 % DHA in total fatty acids (TFA) (de Swaaf *et al.*, 2003). The highest overall DHA productivity so far reported for this organism is 53 $mg\ l^{-1}\ h^{-1}$. This organism possesses the unique advantage of producing DHA with other PUFA present at less than 1 % of TFA. Therefore, SCO from *C. cohnii* clinical trials obtained the GRAS (generally recognized as safe) status for human consumption, while *Schizochytrium* sp. only possess the GRAS status for animal nutrition (Ward and Singh, 2005). Martek Biosciences Corp. exclusively produced *C. cohnii* to be implemented as pre-term and full-term infant formulae additives.

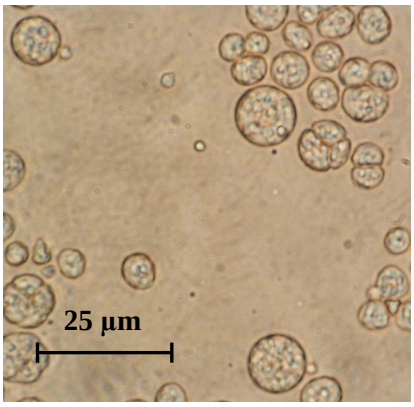
Microbial oils are gaining importance as nutraceutical ingredients and pharmacy-grade products. Microbial lipids are included as functional foods in daily consumed products such as milk (Franklin *et al.*, 1999) and eggs (Lewis *et al.*, 2000). In 2004, Nutrinova and Martek Biosciences absorbed 19 % of the omega-3 nutraceutical market using microbial oils as additives to infant formulae (FPD, 2006). In aquaculture, microbial sources could be effectively used in the culture of fish larvae to cover the high nutritional demand during these early stages. For instance, microbial biomass is implemented as live prey enrichment materials, mainly by two production companies: Aquafauna



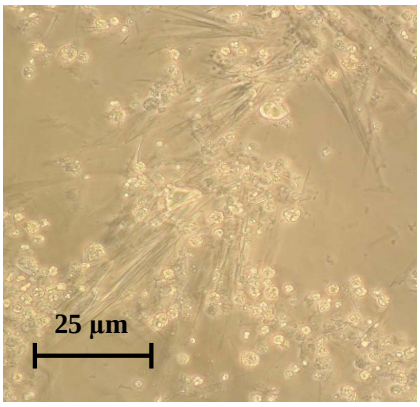
(a) Optical microphotographs of *Schizochytrium* G13/2S



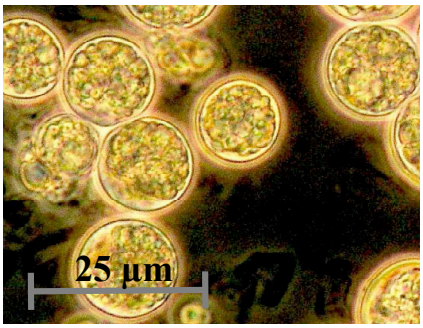
(b) Electron microphotographs of *Schizochytrium* G13/2S



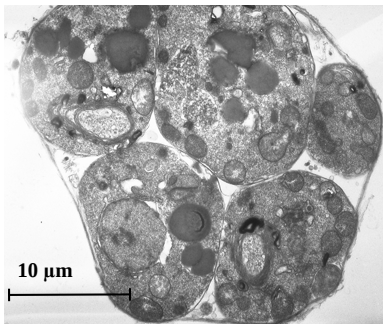
(c) *Schizochytrium* G13/2S dividing cells.



(d) *Schizochytrium*'s ectoplasmic net.



(e) Optical microphotographs of *Crypthecodinium cohnii*.



(f) Electron micrographs of *Crypthecodinium cohnii*

Figure 1.6: Microphotographs of DHA producing microorganisms.

Bio-Marine, Inc. and Advanced BioNutrition, Inc.

1.8. HUFA producing microorganisms in aquaculture

Single cell heterotrophs provide sources of HUFA that can be easily combined to obtain the required dietary fatty acid profile either in live preys (as enrichment material) or be directly implemented into microparticulate diets. So far, most single cell heterotrophs are used for marine fish larviculture rather than as fish oil substitute in the bulk adult aquafeeds. Larval feeds require higher HUFA contents, which allow and justifies the inclusion of a more expensive but better controlled HUFA source. The DHA: EPA ratio of most commercially available fish oils is circa 10: 20 while the requirements of marine fish larvae are predominantly for DHA. During enrichment, the optimal DHA: EPA ratio is further compromised. Enrichment protocols are performed in highly aerated conditions for 12–24 *h*, where the fish oil emulsions are susceptible to peroxidation (Mc Evoy *et al.*, 1995). Besides, *Artemia*, which contains some EPA but no DHA (Navarro *et al.*, 1993), is able to retro-convert the DHA of the enrichment emulsion to EPA (Izquierdo, 1988; Evjemo *et al.*, 2001). As a consequence, the initial DHA:EPA ratio of the enrichment should be very high in order to maintain the nutritional quality of the prey throughout the enrichment and following starvation. This ratio is not provided by common fish oil. Alternatively, the marine microheterotrophs *Schizochytrium* sp. and *C. cohnii* are good sources of DHA. Dried-whole-cell preparations of these organisms may be directly administered to live prey, providing a naturally protected lipid source that includes antioxidants, a quantity of polar lipids as well as proteins and micronutrients. Since EPA is below 2 % TFA in those microorganisms, they could provide and maintain the required DHA:EPA ratio over the enrichment period. In addition, *Mortierella* spp. fungi are good sources of ARA.

Biomass derived from microheterotrophs has been successfully implemented as live prey enrichment for Atlantic cod (*Gadus morhua*) (Cutts *et al.*, 2006; Park *et al.*, 2006), haddock (*Melanogrammus aeglefinus*) (Blair *et al.*, 2003), cobia (*Rachycentron canadum*) (Faulk and Holt, 2005), striped trumpeter (*Latris lineata*) (Battaglene *et al.*, 2006), yellowtail flounder (*Limanda ferruginea*) (Copeman *et al.*, 2002), Atlantic halibut (*Hippoglossus hippoglossus*) (Shields and Irwin, 1998), striped bass (*Morone saxatilis*), European sea bass (*Dicentrarchus labrax*) and gilthead seabream (Harel *et al.*, 2002; Koven *et al.*, 2001). *Schizochytrium* sp. or *C. cohnii* enrichment of *Artemia* (Ritar *et al.*, 2004) and rotifers (*Brachionus plicatilis*) (Park *et al.*, 2006) increased DHA content to the required DHA:EPA ratio. Live prey,

especially *Artemia*, retro-converts DHA to EPA, hence, *a priori*, there is no need to implement other microbial EPA enrichments. Fish larvae generally obtain better growth with the enrichment based on microbial DHA sources due to the high DHA:EPA ratios attained in live prey in comparison with other commercial enrichments (Battaglene *et al.*, 2006; Cutts *et al.*, 2006; Harel *et al.*, 2002). In addition, *Schizochytrium*-based enrichment provides n-6 DPA, which is partially retro-converted to ARA by rotifer and by *Artemia* (Barclay and Zeller, 1996). This is usually reflected in the ARA content of larvae and is associated with better survival (Koven *et al.*, 2001; Park *et al.*, 2006). Algamac® (*Schizochytrium*-containing commercial product), due to the increased bacterial load over the fish oil emulsions and live algae enrichments, induced a higher mortality in *Artemia*, but this did not transcend to the larvae performance (Ritar *et al.*, 2004).

The inclusion of SCO directly in microparticulate diets should avoid the partial loss of material during live prey enrichment. Although industrial hatcheries still rely on live prey feeds, microdiets will ideally provide feeds containing better controlled nutritional quality, thus leading to more predictable fry productions. Algamac® produced from *Schizochytrium* sp. was implemented during seabream first-feeding microdiets (Robin and Peron, 2004) enhancing DHA incorporation by larvae. In addition, dietary fish oil was substituted with 2 and 4 % dry weight of *C. cohnii* biomass in seabream fingerlings showing a better growth and survival (Atalah *et al.*, in press). *Schizochytrim* oil has been used as fish oil replacement in Atlantic salmon parr diets (Miller *et al.*, in press). The biomass of *Thraustochytrid* ACEM 6063, a *Schizochytrium* related organism, was implemented in rapeseed oil-based diets for Atlantic salmon juveniles (Carter *et al.*, 2003). The complete substitution of fish oil did not alter acceptance, growth or immune function, but it increased the cumulative mortality following transfer to seawater and challenge to *Vibrio anguillarum* pathogen. Defatted biomass of *Mortierella alpina* was introduced in striped bass (*Morone saxatilis*) broodstock diets improving egg hatching (Harel *et al.*, 2002).

1.9. Gilthead seabream as a model to study the effect of heterotrophic organisms as a source of HUFA

Seabream is a marine teleost (family *Sparidae*) that traditionally has been cultured extensively in saltwater coastal lagoons of the Mediterranean basin (e.g. Cadiz Bay). However, it was not until the early eighties that this finfish has been cultured intensively. Since then, world production has increased to 91100 tons in 2005 according to the Federation of European Aquaculture Producers (FEAP) (Figure 1.7). Currently, seabream production constitutes

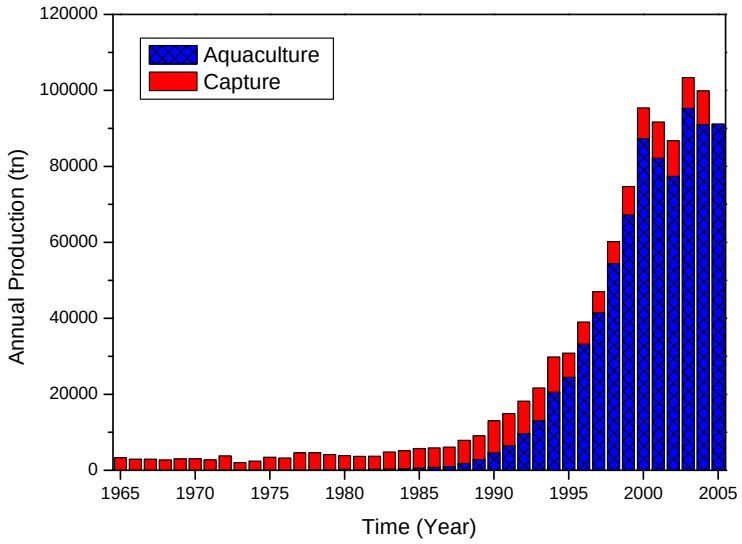


Figure 1.7: Global production of gilthead seabream. Based on FAO (2004) statistics.

the third most cultivated species by volume in Europe after salmon and trout. This consolidated industry produced 92 % of marketed seabream in 2003, the remaining 8 % being captured from the wild. Main producing countries in 2005 are Greece (55 %), Spain (17 %), Turkey (17 %) and Italy (9 %) (Apromar, 2006).

Gilthead seabream cultivation techniques are well developed and their nutritional requirements are used as a model to developed feeds for other less developed species such as the European sea bass (*Dicentrarchus labrax*) or the red porgy (*Pagrus pagrus*). Particularly the lipid metabolism is well studied and their requirements are detailed for each of its life stages. Because the larval stage is nutritionally the most delicate, it requires an accurate dietary fatty acid profile. For these reasons, gilthead seabream are ideal finfish to evaluate the performance of microbial sources as fish oil substitutes in aquafeeds.

1.10. Objectives

In view of the importance of HUFA, particularly DHA, for marine fish larvae and the constraints in fish oil supply as a source of these fatty acids, the purpose of this thesis was to test the viability of microbial heterotrophic organisms as alternative sources of essential fatty acids for the development of sustainable aquaculture of finfish. To achieve that goal two heterotrophic

microalgae, *Schizochytrium* sp. and *Crypthecodinium cohnii*, were selected (due to the advantages mentioned above) as alternative sources of DHA for gilthead seabream larvae. In order to develop an integrated process several specific objectives were addressed:

1. To study the physiology and biochemistry of lipid accumulation in *Schizochytrium* sp. and *C. cohnii* under different medium, culture and aerating conditions.

2. To optimize the fermentation of both microorganisms, implementing the culture parameters and techniques that modulate lipid biochemistry and ultimately DHA accumulation.

3. To evaluate the potential of fermentation technology in aquaculture and facilitate the implementation of this facilities.

4. To determine the effect fish oil replacement by *Schizochytrium* sp. and *C. cohnii* biomass in microdiets for gilthead seabream, examining dietary intake and larval growth and survival.

5. To test the effect of both microalgae in larval health, including stress and disease resistance.

6. To analyze the effect of providing both species of microalgae on larval lipid metabolism, studying the changes in larval fatty acid profiles.

References

Ackman, R.G., Jangaard, P.M., Hoyle, R.J. and Brokerhoff, H. (1964) Analyses of the fatty acids produced by the diatom *Skeletonema costatum*. *J Fish Res Board Can*, 21:747–756.

Apromar (2006) Informe anual: La acuicultura marina de peces en España. Technical report, Asociación Empresarial de Productores de Cultivos Marinos www.apromar.es.

Apt, K.E. and Behrens, P.W. (1999) Commercial developments in microalgal biotechnology. *J Phycol*, 35:215–226.

Atalah, E. Hernández-Cruz, C.M., Izquierdo, M.S., Rosenlund, G., Caballero, M.J., Valencia, A. and Robaina, L. (In press) Two microalgae *Crypthecodinium cohnii* and *Phaeodactylum tricornutum* as alternative source of essential fatty acids in starter feeds for seabream (*Sparus aurata*). *Aquaculture*, 270(1–4):178–185.

Barclay, W.R., Bailey, R.B., DiMasi, D., Hansen, J.M., Mirrasoul, P.J., Ruecker, C.M., Veeder III, G.T. and Kaneko, T. (2003) Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermentors. United States Patent: 09,771,352, *Martek Biosciences Corporation* Boulder, Colorado, pp. 1–12.

Barclay, W. and Zeller, S. (1996) Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. *J World Aquacult Soc*, 27(3):314–322.

Barclay, W.R., Meager, K.M. and Abril, J.R. (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J Appl Phycol*, 6:123–129.

Barclay, W.R., Terry, K.L., Nagle, N.J., Weissman, J.C. and Goebel, R.P. (1987) Potential of new strains of marine and inland saline-adapted microalgae for aquaculture. *J World Aquacult Soc*, 18:218–228.

Battaglene, S.C., Morehead, D.T., Cobcroft, J.M., Nichols, P.D., Brown, M.R. and Carson, J. (2006) Combined effects of feeding enriched rotifers and antibiotic addition on performance of striped trumpeter (*Latris lineata*) larvae. *Aquaculture*, 251(1–2):456–471.

Bell, J.G., McEvoy, L.A., Estévez, A., Shields, R.J. and Sargent, J.R. (2003) Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture*, 227(1–4):211–220.

Bell, J.G., Torstensen, B. and Sargent, J.R. (2005) Replacement of marine fish oils with vegetable oils in feeds for farmed salmon. *Lipid Tech*, 17:7–11.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C. and Sargent, J.R. (1995) Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids*, 30(5):443–449.

Beney, L. and Gervais, P. (2001) Influence of the fluidity of the membrane on the response of organisms to environmental stresses. *Appl Microbiol Biotech*, 57:34–42.

Benítez-Santana, T., Masuda, R., Juarez-Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M. and Izquierdo, M.S. (2006) Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. *Aquaculture*, 34(1):100–117.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M. and Fernández-Palacios, H. (1999) Effect of dietary arachidonic acid levels on growth and survival of gilthead sea bream (*Sparus aurata*) larvae. *Aquaculture*, 127(1–4):265–276.

Bistrian, B.R. (2003) Clinical aspects of essential fatty acid metabolism: Jonathan Rhoads lecture. *J Parent Ent Nutr*, 27(3):168–175.

Blair, T., Castell, J., Neil, S., D'Abramo, L., Cahu, C.L., Harmon, P. and Ogunmoye, K. (2003) Evaluation of microdiets versus live feeds on growth, survival and fatty acid composition of larval haddock (*Melanogrammus aeglefinus*). *Aquaculture*, 225(1–4):451–461.

Borowitzka, M.A. (1997) Microalgae for aquaculture: opportunities and constraints. *J Appl Phycol*, 9(5):393–401.

Brenna, J.T. (2002) Efficiency of conversion of α-linolenic acid to long chain n-3 fatty acids in man. *Curr Opin Clin Nutr*, 5(2):127–132.

Brenner, R.R. (2003) Hormonal modulation of Δ^6 and Δ^5-desaturases: case of diabetes. *Prostag Leukotr Ess*, 68(2):151–162.

Broadhurst, C.L. and Wang, Y. (2002) Brain-specific lipids from marine, lacustrine, or terrestrial food resources: potential impact on early African *Homo sapiens*. *Comp Biochem Phys B*, 131(4):653–673.

Brown, M.F. (1994) Modulation of rhodopsin function by properties of the membrane bilayer. *Chem Phys Lipids*, 73(1–2):159–180.

Brugére, C. and Ridler, N. (2004) Global aquaculture outlook in the next decades: an analysis of national aquaculture production forecasts 2030. *Fisheries Circular 1001*, FAO, Rome.

Burdge, G.C. and Wootton, S.A. (2002) Conversion of α-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *Brit J Nutr*, 88(4):411–420.

Burgess, J.R., Stevens, L., Zhang, W. and Peck, L. (2000) Long-chain polyunsaturated fatty acids in children with attention-deficit hyperactivity disorder. *Am J Clin Nutr*, 71(1):327–330.

Buzzi, M., Henderson, R.J. and Sargent, J.R. (1997) Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. *Comp Biochem Phys B*, 116(2):263–267.

Cahu, C.L., Zambonino-Infante, J.L. and Takeuchi, T. (2003) Nutritional components affecting skeletal development in fish larvae. *Aquaculture*, 227(1–4):254–258.

Calder, P.C. (2002) Dietary modification of inflammation with lipids. *P Nutr Soc*, 61(3):345–358.

Calder, P.C., Yaqoob, P., Thies, F., Wallace, F.A. and Miles, E.A. (2002) Fatty acids and lymphocyte functions. *Brit J Nutr*, 87(1):31–48.

Carter, C.G., Bransden, M.P., Lewis, E.T. and Nichols, P.D. (2003) Potential of thraustochytrids to partially replace fish oil in Atlantic salmon feeds. *Mar Biotechnol*, 5:480–492.

Cavalier-Smith, T., Allsopp, M.T.E.P. and Chao, E.E. (1994) Thraustochytrids are chromists, not fungi: 18S rRNA signatures of heterokonta. *Philos T Royal Soc B*, 348(1318):387–397.

Chen, C. and Bazan, N.G. (2005) Lipid signaling: sleep, synaptic plasticity, and neuroprotection. *Prost Oth Lipid M*, 77:65–76.

Cleland, L.G., James, M.J. and Proudman, S.M. (2003) The role of fish oils in the treatment of rheumatoid arthritis. *Drugs*, 63(9):845–853.

Cole, G.M., Lim, G.P., Yang, F., Teter, B., Begum, A., Ma, Q., Harris-White, M.E. and Frautschy, S.A. (2005) Prevention of Alzheimer's disease: ω-3 fatty acid and phenolic anti-oxidant interventions. *Neurobiol Aging*, 26(1):133–136.

Colwell, R.R. (1997) Microbial biodiversity and biotechnology. In: Colwell (Ed.), Biodiversity II: understanding and protecting our biological resources. *Joseph Henry Press of the National Academy Press*, Washington DC., pp. 279–287.

Connor, W.E. (2000) Importance of n-3 fatty acids in health and disease. *Am J Clin Nutr*, 71(1):171–175.

Copeman, L.A., Parrish, C.C., Brown, J.A. and Harel, M. (2002) Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. *Aquaculture*, 210(1):285–304.

Crawford, M.A., Bloom, M., Broadhurst, C.L., Schmidt, W.F., Cunnane, S.C., Galli, C., Gehbremeskel, K., Linseisen, F., Lloyd-Smith, J. and Parkington, J. (1999) Evidence for the unique function of docosahexaenoic acid during the evolution of the modern hominid brain. *Lipids*, 34:39–47.

Cunnane, S.C. (2000) The conditional nature of the dietary need for polyunsaturates: a proposal to reclassify essential fatty acids as conditionally-indispensable or conditionally-dispensable fatty acids. *Brit J Nutr*, 84(6):803–812.

Cutts, C.J., Sawanboonchun, J., Mazorra de Quero, C. and Bell, J.G. (2006) Diet-induced differences in the essential fatty acid (EFA) compositions of larval Atlantic cod (*Gadus morhua* L.) with reference to possible effects of dietary EFAs on larval performance. *ICES J Mar Sci*, 63(2):302–310.

de Swaaf, M.E., Pronk, J.T. and Sijtsma, L. (2003) Fed-batch cultivation of the docosahexaenoic-acid-producing marine alga *Crypthecodinium cohnii* on ethanol. *Appl Microbiol Biotechnol*, 61(1):40–43.

Emken, E.A., Adolf, R.O. and Gulley, R.M. (1994) Dietary linoleic acid influences desaturation and acylation of deuterium-labelled linoleic and linolenic acids in young adult males. *Biochim Biophys Acta*, 1213(3):277–288.

Evjemo, J.O., Danielsen, T.L. and Olsen, Y. (2001) Losses of lipid, protein and n-3 fatty acids in enriched *Artemia franciscana* starved at different temperatures. *Aquaculture*, 193(1–2):65–80.

FAO (2004) Collation, analysis and dissemination of global and regional fishery statistics. Fi programme websites http://www.fao.org/figis/ [Accessed Dec 15 2006]., Fishery Information, Data and Statistics Unit (FAO-FIDI), Rome, pp. 144.

FAO/WHO (1994) Fats and oils in human nutrition. Joint expert consultation. Food and Nutrition, 57, (FAO/WHO) World Health Organisation, Rome, pp 120.

Faulk, C.K. and Holt, G.J. (2005) Advances in rearing cobia *Rachycentron canadum* larvae in recirculating aquaculture systems: live prey enrichment and greenwater culture. *Aquaculture*, 249(1–4):231–243.

Franklin, S.T., Martin, K.R., Baer, R.J., Schingoethe, D.J. and Hippen, A.R. (1999) Dietary marine algae (*Schizochytrium* sp.) increases concentrations

of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J Nutr*, 129(11):2048–2054.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S. (2005) Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). *Comp Biochem Physiol B*, 142(4):410–418.

Ganga, R., Tort, L., Acerete, L., Montero, D. and Izquierdo, M.S. (2006) Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*. *J Endocrin*, 190:39–45.

Gerster, H. (1998) Can adults adequately convert α-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int J Vit Nutr Res*, 68(3):159–173.

Gill, C.O., Hall, M.J. and Ratledge, C. (1977) Lipid-accumulation in an oleaginous yeast (*Candida* 107) growing on glucose in single-stage continuous culture. *Appl Environ Microb*, 33(2):231–239.

Giraud, M.N., Motta, C., Boucher, D. and Grizard, G. (2000) Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. *Hum Reprod*, 15(10):2160–2164.

Gladue, R.M. and Maxey, J.E. (1994) Microalgal feeds for aquaculture. *J Appl Phycol*, 6(6):131–141.

Granger, L.M., Perlot, P., Goma, G. and Pareilleux, A. (1993) Effect of various nutrient limitations on fatty-acid production by *Rhodotorula glutinis*. *Appl Microbiol Biotech*, 38(6):784–789.

Hansen, H.S., Jensen, B. and von Wettstein-Knowles, P. (1986) Apparent in vivo retro-conversion of arachidonic acid to linoleic acid. *Biochim Biophys Acta*, 878(2):284–287.

Harel, M., Koven, W., Lein, I., Bar, Y., Behrens, P., Stubblefield, J., Zohar, Y. and Place, A.R. (2002) Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs. *Aquaculture*, 213(2):347–362.

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawa, S. and Shimizu, S. (1998) Enhancement of arachidonic acid production by *Mortierella alpina* 1S–4. *J Am Oil Chem Soc*, 75(11):1471–1586.

Hites, R.A., Foran, J.A., Carpenter, D.O., Hamilton, M.C., Knuth, B.A. and Schwager, S.J. (2004) Global assesment of organic contaminants in farmed salmon. *Science*, 303:226–229.

Hong, S., Gronert, K., Devchand, P.R., Moussignac, R.L. and Serhan, N.C. (2003) Novel docosatrienes and 17s-resolvins generated from docosahexaenoic acid in murine brain, human blood and glial cells. *J Biol Chem*, 278(17):14677–14687.

Ibeas, C., Cejas, J.R., Fores, R., Badía, P., Gómez, T. and Hernández, A.L. (1997) Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream (*Sparus aurata*) juveniles. *Aquaculture*, 150(1–2):91–102.

Iijima, N., Tanaka, S. and Ota, Y. (1998) Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. *Fish Physiol Biochem*, 18(1):59–69.

Izquierdo, M.S. (1988) Essential fatty acid requirements for marine fish larvae. PhD thesis, La Laguna University, Spain, pp. 255.

Izquierdo, M.S. (1996) Essential fatty acid requirements of cultured marine fish larvae. *Aquacult Nutr*, 2(2):183–191.

Izquierdo, M.S. (2005) Essential fatty acid requirements in Mediterranean fish species. *Cah Options Mediterr*, 63:91–102.

Izquierdo, M.S., Fernández-Palacios, H. and Tacon, A.G.J. (2001) Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture*, 197(1–4):25–42.

Izquierdo, Montero, D., Robaina, L., Caballero, M.J., Rosenlund, G. and Ginés, R. (2005) Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. *Aquaculture*, 250:431–444.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L. and Rosenlund, G. (2003) Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. *Aquacult Nutr*, 9(6):397–407.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernández-Cruz, C. (2000) Recent advances in lipid nutrition in fish larvae. *Fish Physiol Biochem*, 22:97–107.

James, M.J., Ursin, V.M. and Cleland, L.G. (2003) Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am J Clin Nutr*, 77(5):1140–1145.

Jeffrey, B.G., Weisinger, H.S., Neuringer, M. and Mitchell, D.C. (2001) The role of docosahexaenoic acid in retinal function. *Lipids*, 36(9):859–871.

Josupeit, H. (2006) Fish oil production disappointing. Fish info network market report, FAO Globefish, Rome, pp. 2.

Kemperman, R.F., Veurink, M., van der Wal, T., Knegtering, H., Bruggeman, R., Fokkema, M.R., Kema, I.P., Korf, J. and Muskiet, F.A. (2006) Low essential fatty acid and B-vitamin status in a subgroup of patients with schizophrenia and its response to dietary supplementation. *Prostag Leukotr Ess*, 74(2):75–85.

Kennedy, M.J., Reader, S.L. and Davies, R.J. (1993) Fatty acid production characteristics of fungi with particular emphasis on γ-linolenic acid production. *Biotech Bioeng*, 42(5):625–634.

Khaware, R.K., Koul, A. and Prasad, R. (1995) High membrane fluidity is related to NaCl stress in *Candida membranefaciens*. *Biochem Mol Biol Int*, 4:875–880.

Kingsella, J.E., Lokesh, B., Broughton, S. and Whelan, J. (1990) Dietary polyunsaturated fatty acids and eicosanoids: potential effects on the modulation of inflamatory and immune cells: an overview. *Nutrition*, 6(1):24–62.

Klein, H.P. and Volkmann, C.M. (1975) Factors affecting palmitoylcoenzyme A desaturase of *Saccharomyces cerevisiae*. *J Bacteriol*, 124:718–723.

Koven, W.M., Kissil, G.W. and Tandler, A. (1989) Lipid and n-3 requirement of *Sparus aurata* larvae during starvation and feeding. *Aquaculture*, 79:185–191.

Koven, W., Kolkovski, S., Hadas, E., Gamsiz, K. and Tandler, A. (2001) Advances in the development of microdiets for gilthead seabream, *Sparus aurata*: a review. *Aquaculture*, 194(1–2):107–121.

Kris-Etherton, P.M., Harris, W.S. and Appel, L.J. (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*, 106(1):2747–2757.

Kris-Etherton, P.M., Taylor, D.S., Yu-Poth, S., Huth, P., Moriarty, K., Fishell, V., Hargrove, R.L., Zhao, G. and Etherton, T.D. (2000) Polyunsaturated fatty acids in the food chain in the United States. *Am J Clin Nutr*, 71(1):179–188.

Kyle, D.J. and Gladue, R.M. (1993) Eicosapentaenoic acids and methods for their production. United States Patent 5244921, *Martek Corporation*, Columbia, pp. 60.

Lewis, N.M., Seburg, S. and Flanagan, N.L. (2000) Enriched eggs as a source of n-3 polyunsaturated fatty acids for humans. *Poultry Sci*, 79(7):971–974.

Lichtenstein, A.H. (2003) Dietary fat and cardiovascular disease risk: quantity or quality? *J Women Health Gen B*, 12(2):109–114.

Madsen, L., Rustan, A.C., Vaagenes, H., Berge, K., Dyroy, E. and Berge, R.K. (1999) Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate reference. *Lipids*, 34(9):951–963.

Masuda, R. (2003) The critical role of docosahexaenoic acid in marine and terrestrial ecosystems: from bacteria to human behaviour. In Browman, H.I. and Skiftesvik, A.B., (Eds), *The Big Fish Bang*, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, Bergen, Norway, pp. 249–256.

Masuda, R., Takeuchi, T., Tsukamoto, K., Ishizaki, Y., Kanematsu, M. and Imaizum, K. (1998) Critical involvement of dietary docosahexaenoic acid in the ontogeny of schooling behaviour in the yellowtail. *J Fish Biol*, 53(3):471–484.

Mc Evoy, L.A., Naess, T., Bell, J.G. and Lie, Ø. (1998) Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglossus hippoglossus*) fed enriched *Artemia*: a comparison with fry fed wild copepods. *Aquaculture*, 163(3–4):237–250.

Mc Evoy, L.A., Navarro, J.C., Bell, J.G. and Sargent, J.R. (1995) Autoxidation of oil emulsions during the *Artemia* enrichment process. *Aquaculture*, 134(1–2):101–112.

Meyer, F. and Bolch, K. (1963) Effect of temperatures on the enzymatic synthesis of unsaturated fatty acids in *Torulopsis utilis*. *Biochim Biophys Acta*, 77:671–673.

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

Miller, M.R., Nichols, P.D. and Carter, C.G. (in press) Replacement of fish oil with thraustochytrid *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. *Comp Biochem Physiol A*, DOI 17588797.

Montero, D., Socorro, J., Tort, L., Caballero, M.J., Robaina, L.E., Vergara, J.M. and Izquierdo, M.S. (2004) Glomerulonephritis and immunosuppression associated with dietary essential fatty acid deficiency in gilthead sea bream, *Sparus aurata* L., juveniles. *J Fish Dis*, 27(5):297–306.

Mourente, G. (2003) Accumulation of DHA (docosahexaenoic acid; 22:6n-3) in larval and juvenile fish brain. In: Browman, H.I. and Skiftesvik, A.B. (Eds), *The Big Fish Bang*, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, Bergen, Norway, pp. 139–148.

Mourente, G. and Odriozola, J.M. (1990) Effect of broodstock diets on lipid classes and their fatty acid composition in eggs of gilthead sea bream (*Sparus aurata* l.). *Fish Phys Bioch*, 8(2):93–101.

Müller-Navarra, D.C., Brett, M.T., Liston, A.M. and Goldman, C.R. (2000) A highly- unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature*, 403:74–77.

Müller-Navarra, D.C., Brett, M.T., Sangkyu, P., Chandra, S., Ballantyne, A.P., Zorita, E. and Goldman, C.R. (2004) Unsaturated fatty acid content in seston and tropho-dynamic coupling in lakes. *Nature*, 427(6969):69–72.

Nakamura, M.T. and Nara, T.Y. (2003) Essential fatty acid synthesis and its regulation in mammals. *Prostag Leukotr Ess*, 68(2):145–150.

Nakayama, H., Mitsui, T., Nishihara, M. and Kito, M. (1980) Relation between growth temperature of *E. coli* and phase transition temperatures of its cytoplasmic and outer membranes. *Biochim Biophys Acta*, 601(1):1–10.

Navarro, J.C., Amat, F. and Sargent, J.R. (1993) The lipids of the cysts of freshwater- and marine-type *Artemia*. *Aquaculture*, 109(3–4):327–336.

Naylor, R.L., Goldburg, J.H., Primaver, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenko, J., Mooney, H. and Troell, M. (2000) Effect of aquaculture in world fish supplies. *Nature*, 405:1017–1024.

Nettleton, J.A. (1991) Omega-3 fatty acids: comparison of plant and seafood sources in human nutrition. *J Am Diet Assoc*, 91(3):331–337.

O'Mara, C.J. (1998) U.S. oil seed industry looks at trade issues. *Inform*, 9:132–136.

Owen, J.M., Adron, J.W., Middleton, C., and Cowey, C.B. (1975). Elongation and desaturation of dietary fatty acids in turbot *Scophtalmus maximus* L., and rainbow trout, *Salmo gairdnerii*. *Lipids*, 10(9):528–531.

Park, H.G., Puvanendran, V., Kellett, A.P., Christopher, C. and Brown, J.A. (2006) Effect of enriched rotifers on growth, survival, and composition of larval Atlantic cod (*Gadus morhua*). *ICES J Mar Sci*, 63(2):285–295.

Peyou-Ndi, M.M., Watts, J.L. and Browse, J. (2000) Identification and characterization of an animal Δ-fatty acid desaturase gene by heterologous expression in *Saccharomyces cerevisiae*. *Arch Biochem Biophys*, 376(2):399–408.

Qiu, X., Hong, H.P. and MacKenzie, S.L. (2001) Identification of a Δ^4 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(2):31561–31566.

Rabinovich, A.R. and Ripatti, P.O. (1991) On the conformational, physical properties and functions of polyunsaturated acyl chains. *Biochim Biophys Acta*, 1085(1):53–62.

Rapoport, S.I. and Bosetti, F. (2002) Do lithium and anticonvulsants target the brain arachidonic acid cascade in bipolar disorder? *Arch Gen Psychiat*, 59(7):592–596.

Ratledge, C. (1992) Microbial lipids: commercial realities or academic curiosities. In: Ratledge, C. (Ed), Industrial applications of single cell oils. *AOCS press*, Champaign, Illinois, pp. 1–15.

Ratledge, C. (2002a) Micro-organisms as sources of polyunsaturated fatty acids. In: Gunstone, F.D. (Ed), Structured and modified lipids, *Marcel Dekker, Inc.*, Basel, New York, pp. 351–400.

Ratledge, C. (2002b) Regulation of lipid accumulation in oleaginous micro-organisms. *Biochem Soc Trans*, 30:1047–1050.

Ratledge, C. (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, 86:807–815.

Ratledge, C. and Wynn, J.P. (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbiol*, 51:1–51.

Ristic, V. and Ristic, G. (2003) Role and importance of dietary polyunsaturated fatty acids in the prevention and therapy of atherosclerosis. *Medicinski Pregled*, 56(1–2):50–53.

Ritar, A.J., Dunstan, G.A., Nelson, M.M., Brown, M.R., Nichols, P.D., Thomas, C.W., Smith, E.G., Crear, B.J. and Kolkovski, S. (2004) Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. *Aquaculture*, 239(1–4):351–373.

Robin, J.H. and Peron, A. (2004) Consumption vs. deposition of essential fatty acids in gilthead sea bream (*Sparus aurata*) larvae fed semi-purified diets. *Aquaculture*, 238(1–4):283–294.

Rodríguez, C., Izquierdo, M.S., Lorenzo, A., and Fernández-Palacios, H. (1994a) The effect of image HUFA proportions in diets for gilthead seabream (*Sparus aurata*) larval culture. *Aquaculture*, 124(1–4):124–284.

Rodríguez, C., Pérez, J.A., Badía, P., Izquierdo, M.S., Fernández-Palacios, H. and Hernández, A.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(1–2):9–23.

Rodríguez, C., Pérez, J.A., Díaz, M., Izquierdo, M.S., Fernández-Palacios, H. and Lorenzo, A. (1997) Influence of the EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. *Aquaculture*, 150(1–2):77–89.

Rodríguez, C., Pérez, J.A., Lorenzo, A., Izquierdo, M.S. and Cejas, J.R. (1994b) N-3 HUFA requirement of larval gilthead seabream (*Sparus aurata*) when using high levels of eicosapentaenoic acid. *Comp Biochem Physiol A*, 107(4):693–698.

Roux, M.P., Kock, J.L.F., du Preez, J.C. and Botha, A. (1995) The influence of dissolved oxygen tension on the production of cocoa butter equivalents and γ-linolenic acid by *Mucor circinelloides*. *Syst Appl Microbiol*, 18(3):329–334.

Saito, H., Ishihara, K. and Murase, T. (1997) The fatty acid composition in tuna (bonito, *Euthynnus pelamis*) caught at three different localities from tropics to temperate. *J Sci Food Agric*, 73(1):53–59.

Salhi, M., Kolkovski, S., Izquierdo, M.S. and Tandler, A. (1995) Inclusion of lecithin on polar or neutral lipids high in n-3 HUFA in microdiets for gilthead seabream (*Sparus aurata*) larvae. In: Lavens, P., Jaspers, E., and Roelants, I., (Eds), *Larvi'95*, EAS Special publication, Gent, France, 26:184–187.

Sanders, T.A. (2000) Polyunsaturated fatty acids in the food chain in Europe. *Am J Clin Nutr*, 71(1):176–178.

Sargent, J.R., Mc Evoy, L.A. and Bell, J.G. (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture*, 155(1):117–127.

Sargent, J.R., Mc Evoy, L.A., Estévez, A., Bell, J.G., Bell, M., Henderson, J. and Tocher, D.R. (1999) Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture*, 179(1):217–229.

Sargent, J.R. and Tacon, A.G.J. (1999) Development of farmed fish: a nutritionally necessary alternative to meat. *P Nutr Soc*, 58(2):377–383.

Sargent, J.R., Tocher, D.R. and Bell, J.G. (2003) The lipids In: Halver, J. and Hardy, R. (Eds), Fish Nutrition, *Academic press*, San Diego, California, 3^{th} edition, pp. 181–260.

Serhan, N.C., Hong, S., Gronert, K., Colgan, S.P., Devchand, P.R., Mirick, G. and Moussignac, R.L. (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transfirmation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med*, 196(8):1025–1037.

Shepherd, C.J., Pike, I.H. and Barlow, S.M. (2005) Sustainable feed resources of marine origin. In: *Aquaculture Europe 2005*, EAS Special Publication, 35:59–66.

Shields, R.J. and Irwin, S. (1998) Preliminary evaluation of the *Schizochytrium*-based products, Algamac-3010 and Schizotein, as diet enrichments for Atlantic halibut larvae. Technical report, *Aquafauna BioMarine Inc.*, Algyll, UK.

Sijtsma, L. and de Swaaf, M.E. (2004) Biotechnological production and applications of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. *Appl Microbiol Biotech*, 64(2):146–153.

Sijtsma, L., Springer, J., Meesters, P.A.E.P., de Swaaf, M.E. and Eggink, G. (1998) Recent advances in fatty acid synthesis in oleaginous yeast and microalgae. *Recent Res Dev Microbiol*, 2:219–232.

Simopoulos, A.P. (1999) Essential fatty acids in health and chronic disease. *Am J Clin Nutr*, 70(3):560–569.

Simopoulos, A.P. (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr*, 21(3):495–505.

Singh, A., Wilson, S. and Ward, O.P. (1996) Docosahexaenoic acid (DHA) production by *Thraustochytrium* sp. ATCC 20892. *World J Microbiol Biotech*, 12(1):76–81.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M. and Herndl, G.J. (2006) Microbial diversity in the deep sea and the underexplored rare biosphere. *World J Microbiol Biotech*, 103(32):12115–12120.

Sontrop, J. and Campbell, M.K. (2006) Omega-3 polyunsaturated fatty acids and depression: a review of the evidence and a methodological critique. *Prev Med*, 42(1):4–13.

Sprecher, H. (2002) The roles of anabolic and catabolic reactions in the synthesis and recycling of polyunsaturated fatty acids. *Prostag Leukotr Ess*, 67(2–3):79–83.

Sprecher, H.l., Luthria, D., Mohammed, B.S. and Baykousheva, S.P. (1995) Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J Lipid Res*, 36(12):2471–2477.

Staff reporter (2006) Global fish oil production down in 2005. *Food Production Daily*: www.foodproductiondaily.com.

Stillwell, W. and Wassall, S.R. (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chem Phys Lipids*, 126(1):1–27.

Tacon, A. (2004) Use of fish meal and fish oil in aquaculture: a global perspective. *Aq Res Cult Dev*, 1(1):3–14.

Terry, P.D., Rohan, T.E. and Wolk, A. (2003) Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. *Am J Clin Nutr*, 77(3):532–543.

Tocher, D.R., Agaba, M., Hastings, N. and Teale, A.J. (2003) Biochemical and molecular studies of the polyunsaturated fatty acid desaturation pathway in fish. In: Browman, H.I. and Skiftesvik, A.B. (Eds), *The Big Fish Bang*, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, Bergen, Norway, pp. 211–227.

Tocher, D.R. and Ghioni, C. (1999) Fatty acid metabolism in marine fish: low activity of Δ^6 desaturation in gilthead sea bream (*Sparus aurata*) cells. *Lipids*, 34(5):433–440.

Van Anholt, R.D., Spanings, F. A.T., Koven, W.M., Nixon, O., and Wendelaar Bonga, S.E. (2004) Arachidonic acid reduces the stress response of gilthead seabream *Sparus aurata* L. *J Exp Biol*, 207(19):3419–3430.

Voss, A., Reinhart, M., Sankarappa, S. and Sprecher, H. (1991) The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a Δ^4-desaturase. *J Biol Chem*, 266(30):19995–20000.

Wainwright, P.E. (2002) Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. *Proc Nutr Soc*, 61(1):61–69.

Ward, O.P. and Singh, A. (2005) Omega-3/6 fatty acids: alternative sources of production. *Process Biochem*, 40(12):3627–3652.

Watanabe, T. (1982) Lipid nutrition in fish. *Comp Biochem Physiol B*, 73(1):3–15.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S. and Kitajima, C. (1989) Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. *Nippon Suisan Gakk*, 55(9):1635–1640.

Watanabe, T. and Kiron, V. (1994) Prospects in larval fish dietetics. *Aquaculture*, 124(1–4):223–251.

Watanabe, T., Kitajimab, C. and Fujitab, S. (1983) Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture*, 34(1–2):115–.143.

Whelan, J. (1996) Antagonistic effects of dietary arachidonic acid and n-3 polyunsaturated fatty acids. *J Nutr*, 126(4):1086–1091.

Yaguchi, T., Tanaka, S., Yokochi, T., Nakahara, T. and Higashihara, T. (1997) Production of high yields of docosahexaenoic acid by *Schizochytrium* sp. strain SR21. *J Am Oil Chem Soc*, 74:1431–1434.

Yavin, E., Brand, A. and Green, P. (2002) Docosahexaenoic acid abundance in the brain: a biodevice to combat oxidative stress. *Nutr Neurosc*, 5(3):149–157.

Zaslavskaia, L.A., Lippmeier, J.C., Shih, C., Ehrhardt, D., Grossman, A.R. and Apt, K.E. (2001) Trophic obligate conversion of an photoautotrophic organism through metabolic engineering. *Science*, 292(5524):2073–2075.

Chapter 2

General materials and methods

The following dissertation is structured in two major experimental subjects regarding microbial fermentation and fish larvae nutrition. The fermentation work was carried out at the Lipid Research Centre, at the Department of Biological Science of the University of Hull (England). The experiments on gilthead seabream *Sparus aurata* larval nutrition were conducted at the Marine Cultures facilities of the Grupo de Investigación en Acuicultura (ULPGC & ICCM) facilities (Spain). The following chapter explains in detail the general methodology used in these experiments. Particular experimental conditions are specified in each chapter.

2.1. Microbial cultures

Strain maintenance and medium specifications

Schizochytrium sp. strain G13/2S, derived from G13 (Graham Bremer, University of Portsmouth) and deposited at Banco Nacional de Algas (BNA 40–004) (Taliarte, Spain), was preserved (2–3 months) in screw-capped tubes (10 ml capacity) at 10 °C before inoculating (10 % v/v) the starting cultures. The initial medium contained ($g\ l^{-1}$): sea salts (27), glucose (40), proteose peptone (8) and yeast extract (5); pH adjusted to 7 ±0.3 with KOH (1 M).

Further cultures were grown in a defined medium modified from Ashford *et al.* (2000) containing ($g\ l^{-1}$): glucose (40), NaCl (12.5), glutamic acid monosodium salt (4), $MgSO_4\ 7H_2O$ (2.5), KCl (0,5), $CaCl_2$ (0.1), KH_2PO_4 (0.5), unless stated otherwise. This medium also contained trace-element solution (5 $ml\ l^{-1}$) and vitamin solution (1 $ml\ l^{-1}$). The trace element solution contained ($g\ l^{-1}$): EDTA di-sodium salt (6.0), $FeCl_3\ 6H_2O$ (0.29), H_2BO_3 (6.84), $MnCl_2\ 4H_2O$ (0.86), $ZnCl_2$ (0.06), $CoCl_2\ 6H_2O$ (0.026), $NiSO_4\ 6H_2O$ (0.052), $CuSO_4\ 5H_2O$ (0.002), $Na_2MoO_4\ 2H_2O$ (0.005). The vitamin solution contained ($mg\ l^{-1}$): thiamine (100), biotin (0.5) and cyanocobalamin (0.5).

Crypthecodinium cohnii strain ATCC 50060 was maintained in screw-capped tubes, as described before, in the ATCC 460 A2E6 medium that contained ($g\ l^{-1}$): NaCl (23.48), $MgCl_2\ 6H_2O$ (10.63), glucose (4.5), $NaSO_4$

(3.93), Tris buffer (3), glutamic acid monosodium salt (1.5), $CaCl_2$ (1.11), KCl (0.66), $NaHCO_3$ (0.19), KCr (0.15), $C_3H_7Na_2O_6P$ $5H_2O$ (0.15), $(NH_4)_2SO_4$ (0.05), $SrCl_2$ $6H_2O$ (0.04), H_2BO_3 (0.03), $FeCl_3$ $6H_2O$ (0.01), K_2PO_4 (0.01). This medium also contained trace-element solution (3 $ml\ l^{-1}$) and vitamin solutions (1 $ml\ l^{-1}$). The trace element solution contained ($g\ l^{-1}$): EDTA disodium salt (10.0), H_2BO_4 (10), $MnCl_2$ $4H_2O$ (1.5), $CoCl_2$ $6H_2O$ (0.5), $FeCl_3$ $6H_2O$ (0.5) and $ZnCl_2$ (0.1). The vitamin solution contained ($g\ l^{-1}$): thiamine (1) and biotin (0.003). Further cultures were grown in a medium containing ($g\ l^{-1}$): sea salts (25), glucose (27) and yeast extract (2); pH adjusted to 6.5 $\pm$0.3 with NaOH (1 M).

All medium components were autoclaved (121 °C, 15 min) maintaining glucose in a separate container in order to avoid the "browning" of the medium. The vitamin solution was filter sterilised (pore size, 0.2 μm). Both the vitamin mixture and the glucose were added in sterile conditions after sterilisation.

Inoculum development

Sub-culturing of *Schizochytrium* G13/2S and *Crypthecodinium cohnii* was made in static Erlenmeyer flasks of 50 ml (containing 10 ml medium) followed by 250 ml flasks (containing 50 ml). Each stage of the flask cultivation of *Schizochytrium* G13/2S and *Crypthecodinium cohnii* was maintained for 37 and 5–7 days , respectively. The static-flask cultures were used to inoculate shake-flasks 250 ml cultures (120 rpm) containing 50 ml of defined medium. Either static or orbital shaking incubators were always maintained at 27 °C. The inoculum, obtained from a healthy growing culture, was always 10 % v/v because this size improved the viability of the inoculations. The inoculum development was made in flask cultures with the complex medium previously described for each organism. Shake-flask cultures (24 h) were used to inoculate the fermenters (10 % v/v). The culture purity was checked during sampling and inoculation, 2–3 ml of culture was aseptically removed from the fermentation broth and examined under the optical microscope (X 400 and X 100).

Fermentation set-up

The fermenters were autoclaved (121 °C, 30 min), maintaining glucose separated in the inoculation bottle. Before autoclaving, the pH and O_2 probe were calibrated. The pH calibration was made using standard pH 7.0 and pH 4.0 solutions. The O_2 calibration was made by bubbling a distilled water with N_2 until saturation. Once the dissolved O_2 reading was stabilised, the

reading was calibrated to zero and then air was bubbled through the air inlet. Again, when the reading was stabilised, it was calibrated to 100 %. Prior to calibration procedure, the temperature of the media was adjusted to 27 °C.

Three different type of fermenters with 0.8, 2 and 10 l capacity were used. They were all made of glass and stainless still, cylindrical, flat-bottomed and equipped with bladed turbines. The temperature was maintained at 27 °C using a heater or a water bath through a cooling coil in response to the reading of the thermometer inserted in the temperature-port. The pH was controlled through the pumping in the fermenter of an acid and an alkaline solution in response to the pH prove reading. Acid and alkaline solutions were not autoclaved, they were added after the fermenter was allowed to cool. Before the fermenter was inoculated, a sample of the autoclaved media was taken aseptically to determine the pH of the medium through an external reading. The pH reading on the fermenter console was changed accordingly. Before inoculation, a few droops of antifoam (polypropylene glycol-2000) were manually added using pump and during fermentation, according to an insulated conductivity probe, the same pump operated automatically if necessary. The aeration was previously passed through to filters in line (2 μm). The inoculum was introduced aseptically to the fermenter through the inoculation port. Along fermentation, 20–100 ml of culture was removed aseptically through the sampling port at the required data-points. The residual culture broth trapped in the tubing of the sampling port (from the previous sampling) was systematically discharged. At the end of the culture the volume of the remaining culture was measured in a cylinder (100 ml).

Chemostat fermentation

A fermenter of 2.5 l, containing 2 l working volume, was used for both batch and continuous cultivation of *Schizochytrium* (see Chapter 3). The fermenter (in-house manufactured) was equipped with a vaned-disk impeller. In order to obtain dissolved O_2 concentration above 50 % throughout the whole fermentation, vortex mixing was provided a stirring speed of 1000 rpm while aeration was set to 0.5 vol. air (vol. medium)$^{-1}$ min^{-1}. Foaming was controlled in batch cultures by manual addition of polypropylene glycol-2000 when necessary. Addition of antifoam was not needed during continuous culture. The temperature was set to 27 °C and the pH was maintained at 7 with automatic addition of H_2SO_4 (2 M) or KOH (2 M).

During continuous culture, the volume in the vessel was maintained using an overflow weir. The exact working volume used during continuous culture was previously calculated for the stirring speed applied. Medium was supplied (24 h after inoculation) with a flow inducer at a constant dilu-

tion rate (D) calculated according to the working volume of the fermenter. The flow was checked every 4 h. Other parameters such as dissolved O_2, pH, temperature were monitored continuously. Steady-state conditions were maintained for more than five complete volume changes before sampling ($<$ 100 ml), while dissolved O_2 output was used to detect that any perturbation ($>$ 3 % saturation) happened along the period. Different dilution rates were applied starting from low and going to higher values in order to permit the cells adaptation.

Air limitation

Shake flask culture experiments were always performed in triplicates. Air availability was tested in two different ways in order to exclude the effect of the hydrodynamics in the flask: (1) under different culture medium surface to volume ratio using 50, 150 and 250 ml of medium; (2) with 50 ml of medium either normally aerated or sealed with craft paper in a previously N_2-gassed atmosphere.

Anaerobic fermenter cultures were performed in a laboratory bioreactor (Braun Biolab) with a working capacity of 1 l. The foam was controlled by addition of polypropylene glycol-2000 when necessary and pH 7 was maintained by the automatic addition of 2 M KOH or H_2SO_4. Rushton blade stirrer was set to 500 rpm. Two fermentations were performed simultaneously using both anaerobic and aerobic conditions. The anaerobic fermenter was leak tested under 5 psi of positive pressure before inoculation and after cultivation. In order to ensure anaerobic atmosphere, every two days, the culture was sparged with high-purity-filter-sterilised (99.99 %) N_2 (Engineering and Welding, Hull, UK) for ten minutes and all the silicon-rubber tubing were clipped. The aerobic bioreactor was maintained within the same conditions but with 0.5 vol. air vol. medium^{-1} min^{-1} air income.

Ammonium/pH-auxostat fermentation

Schizochytrium G13/2S was cultivated in a 1 l working capacity bioreactor (Braun Biolab) in which the pH was controlled by the automatic addition of 10 % NH_4OH (10 % w/v). The defined medium described above was modified in order to include 1.64 g $(NH_4)_2SO_4$ l^{-1}. All culture components were autoclaved (121 °C, 30 min), maintaining glucose and $MgSO_4$ $7H_2O$ separately from other medium components to avoid magnesium-ammonium-phosphate precipitation. Dissolved O_2 was maintained over 30 % by manual increase of the stirring speed (rushton blade impellers) from 300 to a maximum of 700 rpm. The aeration rate was always 0.5 vol. air (vol. medium)$^{-1}$

min^{-1}, previously filtered through a 0.2 μm sieve. Addition of antifoam was not necessary during fermentation.

Ethanol fed-batch fermentation

Fermentation was carried in a 10 l bioreactor (Bioflo 3000, New Brunswick). The initial medium contained ($g\ l^{-1}$): yeast extract (10), sea salts (25) and ethanol (5.5). During fermentation, 10 ml of polypropylene glycol-2000 antifoam agent was added to the culture. The pH was maintained at 6.5 $\pm$0.5 by automatic addition of NaOH (2 M) or H_2SO_4 (2 M) while the temperature was set to 27 $\pm$0.5 °C. Stirring speed was programmed to automatically increase (but not decrease) from 200 up to a maximum of 1000 rpm, in response to dissolved O_2 levels below 30 %. The fermenter contained two six-blade turbine impellers and four straight baffles, each of them having a width of 1/10 of the vessel diameter. Once the maximum stirring speed was attained, either the initial airflow was manually increased from 5 to 10 $l\ min^{-1}$ or the airflow was programmed to maintain dissolved O_2 levels above 30 % through the automatic substitution of the air by high purity O_2 (Engineering and Welding, Hull, England).

Dissolved O_2 output (or alternatively the degree of air substitution) was used to evaluate the ethanol demand by the culture. Initially, when the residual ethanol was consumed (dissolved O_2 increasing above 35 % saturation), an initial feeding rate of 0.9 ml ethanol $l^{-1}\ h^{-1}$ was set with a flow inducer. The feeding rate was increased every time that an extra pulse of ethanol was automatically consumed by the culture (dissolved O_2 decreasing below 30 %). The maximum feeding rate attained was maintained until the end of the fermentation. Whenever the dissolved O_2 rose above 35 %, the ethanol feeding was stopped temporally in order to avoid growth inhibition.

Analyses in fermentation

Determination of cell dry weight Samples of 20 ml where harvested by centrifugation (3,000 g) for 5 min at 10 °C. Biomass was washed twice by resuspension in demineralised water (10 °C) and centrifuged as before. The pellet fraction was freeze-dried and dry biomass was measured in a microbalance. The pellet fraction of the second centrifugation was freeze-dried to constant weight and dry biomass contained in a pre-weighted flask was measured in a microbalance.

Fatty acid analysis Fatty acid (FA) analysis of *C. cohnii* freeze-dried biomass (25–30 mg) was made using heptadecanoic acid (2.5 mg per sam-

ple), previously dissolved in methanol: chloroform 3:1, as internal standard. *Schizochytrium* G13/2S biomass (10–15 mg) was analysed using nonadecaenoic acid (1.25 mg per sample) as internal standard previously dissolved in hexane. Fatty acid methylation was conducted by the method of Rodríguez-Ruiz *et al.* (1998). Transesterification reagent (methanol: acetyl chloride 20:1 (v/v)) was prepared freshly on ice. Lyophilised biomass, hexane (500 μl), transesterification reagent and the internal standard were added into a tight screwed test tube and then heated at 100 °C during 10 min. After cooling the tubes at room temperature, reaction was stopped adding 1 ml of distilled water. Two phases were established rapidly, upper one (hexanic) contained the fatty acid methyl esters (FAMEs) and was stored in vials. FAMEs were then analyzed by GC using He as a carrier gas with a BPX70 25 m x 0.22 mm i.d. column (SGE). The temperature of the column was raised from 160 °C to 225 °C at 5 °C min^{-1} and then maintained at 225 °C for further 5 min. Peak detection was performed by a flame ionisation detector. The temperatures of the injection and detection ports were 250 °C. Peaks were identified by comparison with well characterised standards (Sigma, 189.19).

Determination of nitrogen content The supernatant and cell biomass of each sample were freeze-dried and 10 mg was then analyzed by a quantitative dynamic flash combustion method for elementary C, H, N and S detection. Instantaneous oxidation was attained in a 1020 °C combustion reactor supplied with O_2. Excess of O_2 was then reduced through a Cu reduction catalyst layer. The elements were separated in a 2 m chromatographic column packed with Porapak QS of 80/50 mesh size and ran at 60 °C using He as carrier gas. Detection was achieved by thermal conductivity.

Glucose determination The residual glucose concentrations were analysed using the GOD-PERID method (Boehringer Mannheim). The reagent was prepared every two weeks using horseradish peroxidase (0.8 units ml^{-1}), ABTS (1 mg ml^{-1}) and glucose oxidase (10 units ml^{-1}) diluted in a 100 mM phosphate buffer (pH 7). The reactive was always compared with the internal standard provided with the test kit and the blank containing only distilled water. The reagent was allowed to warm at room temperature before utilization and the samples were diluted to concentrations of 50–100 μg ml^{-1}. The reaction was carried out in half-scale cuvettes mixing thoughtfully 0.1 ml of sample dilution with 2.5 ml of reagent. The absorption was measured after 25–50 min in a spectrophotometer at 660 nm.

Ammonia determination The residual ammonia concentrations were analysed using an indophenol-based method (Chaney and Marbatch, 1962). Two reagent were prepared every two months in distilled water: Reagent 1 contained 10 g phenol l^{-1} and 50 mg of $Na_2[Fe(CN)_5NO]$ $2H_2O$ l^{-1} and Reagent 2 contained 5 g NaOH l^{-1} and 0.42 g NaClO l^{-1}. Different standard solutions of $(NH_4)_2SO_4$ were prepared in order to construct the calibration curve. The samples were diluted to 1–5 μg ml^{-1}. The reaction was carried out at room temperature by mixing 1 ml sample, 5 ml of Reagent 1 and 5 ml of Reagent 2 in a vortex mixer. The absorption was measured after 30 min in a spectrophotometer at 625 nm.

2.2. Rotifers

Rotifers, *Brachionus plicatilis*, were cultivated in 800 l cylindrical containers of 1.5 m height. The container was provided with a central aeration system through a porous stone that was maintained at 20 cm from the bottom. The cultures contained a combination of natural seawater (80 %) and freshwater (20 %). Rotifers, strain S-1, of 150–250 μm of length, were inoculated at initial densities of 100 $ind.\ ml^{-1}$. They were fed with 1.2 g fresh yeast extract 10^6 $ind.\ ^{-1}\ d^{-1}$, ration divided in two doses. Every four days the rotifers were carefully filtered (64 μm mesh), rinsed in water, the container was washed and the culture was resuspended in clean water at optimum culture densities of 100–250 $ind.\ ml^{-1}$.

The rotifers were used to fed *Sparus aurata* larvae during green water cultures and also during the first 10 d of microdiet trialing. The rotifers supplemented along the green water cultures were previously enriched (24 h), at densities of 300 rotifers ml^{-1}, with emulsified Selco (Artemia System, Ghent, Belgium) (0.125 g selco l^{-1} ration in two doses). During the microdiet testing yeast-fed rotifers were directly supplemented in a co-feeding regime to the larval tank, providing no source of HUFA.

2.3. Experimental microdiets

Ingredients

- Lyophilized squid meal (Rieber & Son, Bergen, Norway) was selected as a high quality protein source for seabream. Fishmeal has a lower protein digestibility (Fernández-Palacios *et al.*, 1997) and usually contains impurities such as scales and bones, which account to a considerable content of ash (11.4 % dry weight) (Robaina *et al.*, 1995). In turn, squid meal contained around: 13 % lipids, 80 % protein and 6 % ash.

In order to replace all dietary lipids in Trial 2, the squid meal lipids were extracted with chloroform. Squid meal was suspended in three volumes of solvent and agitated for a few minutes. The particules of the mixture were filtered under a vacuum pump and defatted meal was separated from the chloroform fraction. The extraction was repeated three times until the squid meal contained almost no lipids. The chloroform fraction was introduced in a rotary evaporator to obtain the lipid fraction free of chloroform. The squid meal, almost free of lipids, was spread in a laboratory tray and the remaining solvent was evaporated during 12 h at 38 °C.

- Sardine oil (Agramar S.A., Lanzarote, Spain) was selected as a source of n-3 HUFA for control diet.

- Single cell heterotroph biomass from *Schizochytrium sp.* and *C. cohnii* containing over 11 % cell dry weight (CDW) as docosahexaenoic acid (DHA; 22:6 n-3) were implemented as alternative source to fisheries derived oils. *Schizochytrium* G13/2S was produced according to the ammonium/pH-auxostat method (see Chapter 6) and *C. cohnii* ATCC 50060 biomass was produced according to the ethanol fed-batch method (see Chapter 7), both in a 10 l working capacity fermenter (Bioflo 3000, New Brunswick). The algae biomasses were harvested at the stationary phase of the culture by centrifugation (3000 g, 5 min, 10 °C). The biomass was washed twice in demineralised water in order to eliminate the residual medium and decrease the ash content to 1.3–5.3 % CDW. The carotenoid content of the biomass was less than 35.5 μg carotenoids g^{-1} CDW. A fraction of the biomass paste was homogenised through a single pass in a French press (40–50 ml capacity, -20 °C) at 10 $kpsi$ of pressure. Either homogenized or whole-cell biomass was freeze in liquid nitrogen, lyophilized and stores at -20 °C until utilization.

- Pure olive oil (Carbonel S.A., Córdoba, Spain) was used to increase the lipid content of the diets containing single cell heterotrophs to the levels of the control diets.

- Soybean lecithin (Acofarma, Tarrasa, Barcelona, Spain) containing around 50 % polar lipid was used as a source of phospholipids.

- The attractant mixture (3 g 100 g diet^{-1}) as described by Kanazawa *et al.* (1989) contained (g 100 g diet^{-1}): betaine (0.66), inosine-5-monophosphate (0.5), DL-alanine (0.5), L-sodium aspartate (0.33), phenylalanine (0.25), L-valine (0.25), L-serine (0.17), L-tirosine (0.17) and glicine (0.17).

- The vitamin mixture (6 g 100 g diet^{-1}) contained (mg 100 g diet^{-1}): choline chloride (2965.8), inositol (1450.9), nicotinic acid (290.16), ascorbyl polyphosphate (180), α-tocopherol (150), p-aminobenzoic acid (145), calcium panthotenate (101.59), riboflavin (72.53), thiamin HCl (21.77), piridoxyne HCl (17.28), menadione (17.28), astaxanthin (5) cholecalciferol (3.65), retinol acetate (0.18), cyanocobalamin (0.03).

- The mineral mixture (4.5 g 100 g diet^{-1}) as described by Teshima *et al.* (1982) contained (mg 100 g $diet^{-1}$): $C_3H_5O_31/2Ca$ (1617,210), K_2HPO_4 (758,949), $MgSO_4$ $7H_2O$ (677,545), $Ca(H_2PO_4)$ $2H_2O$ (671,610), NaH_2PO_4 H_2O (381,453), NaCl (215,133), $FeC_6H_5O_7$ (146,884), $ZnSO_4$ $7H_2O$ (14,837), $CoSO_4$ $7H_2O$ (10,706), $MnSO_4$ H_2O (2,998), $CuSO_4$ $5H_2O$ (1,247), KI (0,742) and $Al_2(SO_4)_3$ $6H_2O$ (0,693).

- Taurine (Sigma) was introduced as a nutritional additive.

- Fish gelatin was used at 3 g 100 $g^{-}1$ diet as a agglutinant of the mixture.

Preparation

All ingredients were pulverized and mixed in a mortar. The less abundant-dry components were added first and broken in small particles in order to ensure their homogeneous distribution. The minerals, hydrosoluble vitamins and attractants mixtures were prepared in advance and stored in separate plastic bags. The big crystals present in the attractants and minerals mixtures were pulverized in a Cyclotec 1093 sample mill (Rose Scientific Ltd., Stockholm, Sweden) and sieved (1 mm). Minerals were added to the mortar after the hydrosoluble vitamins, the attractants and an equal quantity of squid meal in order to avoid the formation of chemical complex. The liposoluble vitamins were previously dissolved in a small quantity of chloroform, mixed with the oils and added gradually to the mortar mixture. The choline chloride, which is very hygroscopic, was added after the oils. All components were mixed for 15 min and then the gelatin (36 ml g^{-1} diet) was added, previously dissolved in distilled water at 80 °C. The paste was distributed in 34 mm diameter spaghettis on a tray and dried in an oven at 40 °C for 24 h. The dried pellets were grounded in the Ciclotec and sieved (mod. 211, CISA, Barcelona, Spain) to obtain particle size below 200 μm. The microdiets were conserved in hermetic plastic bags at -80 °C under a nitrogen atmosphere.

2.4. Gilthead seabream larviculture

Green water pre-cultures

Fish cultures were developed in filtered (50 μm) natural seawater 36 ± 1 ppm at 20–22 °C. Gilthead seabream (*Sparus aurata*) larvae were obtained from a natural spawning of the broodstock maintained at the ICCM facilities. The eggs were collected into a submerged mesh during 12–24 h by overflow from the broodstock tank containing both male: and females (2:1 proportion). The viable eggs were selected by the precipitation of the non-fecundated eggs and then incubated (24 h) in a well-aerated 2000 l tank. After hatchling, larvae (100 $ind.\ l^{-1}$) were maintained in the tank under a circulating water system (7 water renovations d^{-1}) during the first three days. The fort day, larvae began to open their mouth, the water circuit was closed and larvae were cultured in green water. Every day 20 l of *Nannochloropsis sp.* (30 10^{-6} $cells\ ml^{-1}$) were added and the enriched rotifer density maintained at 15 $ind.\ ml^{-1}$. For this purpose, freshly enriched rotifers were supplied twice a day. The non-ingested rotifers were removed from the tank by filtration, thus avoiding the presence of starved low nutritional quality rotifers in the tank. Larvae were cultured under natural photoperiod (around 10 h) for 16 (Trial 1) or 19 (Trial 2) days until the beginning of the microdiet experiment in which the larvae attained 5.13 ± 0.06 mm standard length ($\pm$SEM), 0.1 ± 0.002 mg dry body weight and 6.03 ± 0.09 mm, 0.15 ± 0.003 mg respectively.

Experimental tank cultures

Larvae were carefully counted and transferred into 15 fibreglass cylindrical tanks (1900 larvae $tank^{-1}$) containing 170 l working volume. UV-sterilized water at 20–22 °C was provided at 250-300 $ml\ min^{-1}$ into the tank and the overflow was filtered through a 300 mm mesh to remove only the non-ingested food. Airflow was maintained constant at 250 $ml\ min^{-1}$ with a bubbles size that avoided excessive turbulence, as well as the small bubble ingestion by larvae. During the 12 h photoperiod (1500 lux) microdiet was manually spread into the surface every 45 min (2 $g\ day^{-1}$) and during the first 10 d of the experiment yeast-fed rotifers (1.5 $ind.\ ml^{-1}$) were also supplied twice, at noon and mid afternoon, in a co-feeding regime. Larvae were fed during 15 (Trial 1) and 21 (Trial 2) days. The tank components and the water surface was siphoned and cleaned with a plastic spatula daily. Every day the water and airflow was regulated and the temperature and pH was measured. All treatments were performed in randomly distributed triplicates.

Health challenge

After 7 *d* of treatment the larvae from Trial 1 were challenged by the parasite *Amylodinium ocellatum*, a protist (Dinoflagellida) that affects seabream hatcheries producing white opaque protuberances in skin and gills, which eventually causes larval mortality. The parasite was inoculated from previously contaminated nets by locating them in the water reservoir that flows equally into the tanks. Parasite affectation was determined by counting the number of lateral protuberances present in 20 larvae tank^{-1}.

Analyses in larviculture

Dietary acceptance was calculated from the micrographs (25 X) of 20 larvae per tank taken at the end of each experiment. The gut area, as well as the area occupied by the diet was calculated through the transparent larvae with the Image-Pro Plus 6.0 software.

Survival was determined by manually counting live larvae at the beginning and at the end of the experiment.

Larval resistance to stress was determined at the end of each experiment through an air-exposure stress test of 20 larvae maintained for 1 *min* on a scoop net. Following the stress larvae were moved to a 2 *l* aerated container in which survival was manually calculated 24 *h* later.

Larval growth was determined by measuring at the beginning, middle and at the end of the trial the standard length of 20 larvae per tank with a profile projector (Nikon V-12, Nikon co., Tokio, Japan). These larvae were then washed in distilled water and stored in an oven (110 °C) until constant weight in order to measure the mean larval dry weight.

Proximate analysis A sample of initial and all surviving larvae at the end of the trials, as well as of yeast-fed rotifers, were washed in distilled water, drained in absorbent paper, deposited in hermetic plastic bags and stored at -80 °C until proximal composition analysis was performed. Larvae were starved for 24 *h* in order to avoid any contribution of the feed to the biochemical body composition.

Dietary and larval moisture, ash and protein content were determined in triplicates according to the Association of Official Analytical Chemists (AOAC) described methods. Moisture was evaporated in an oven at 110 °C

until constant weight and the difference from the initial weight was then calculated as moisture. The ash content was obtained by incineration at 600 °C and calculated as before. The protein content was calculated from the N content according to the Kjeldhal method. The samples were digested at 420 °C in pure H_2SO_4 catalyzed with Me. The sample was distilled with NaOH at 40 % in a Kjeltek System 1003 distiller (Tecator, Höganäs, Switzerland). Finally, the sample was validated with HCl (1 N) to measure N content and protein content was calculated utilizing a nitrogen conversion factor of 6.75.

Lipid analysis The lipids were extracted according to the method of Folch *et al.* (1956). The fresh sample (150–200 mg) was homogenised in 10 ml chloroform: methanol (2: 1) during 5 min. Lipid fraction was separated by centrifugation and the total lipid content was calculated by gravimetry once the solvent in the lipid fraction (chloroform) is completely evaporated. The lipids were dissolved in toluene and fatty acid methyl esters were obtained by transesterification with 1 % H_2SO_4 in methanol (Christie, 2003). The reaction was carried out in dark at 50 °C along 16 h in a N_2 atmosphere. The methylic esters were extracted with hexane: diethyl ether (1: 1, v/v) that was washed with a solution of $KHCO_3$ (2 % w/v) and purified in a NH_2 Set-pak column (Waters, Mildford, Massachusetts, USA) with 4 ml hexane. Fatty acid methyl esters were separated by gas chromatography (GC) using He as a carrier gas with a fused silica, carbowax 20M, 30 m x 0.32 mm i.d. (df = 0.27 m) column (Supelco, Bellefonte, USA). The initial temperature of the column was set to 170 °C for 10 min, then it was raised to 220 °C at 2.5 °C min^{-1} and finally maintained at 215 °C for a further 5 min. Detection was performed by a flame ionisation detector and the peaks were identified with well characterized internal standards (Sigma).

Statistical analysis Results were compared in Sigmastat 3.5 software. One-way ANOVA and Tukey test for multiple mean comparisons was applied after passing normality and equal variance test. When the test failed, Kruskal-Wallis one-way ANOVA was applied on ranks. Significance was accepted at probabilities of 0.05 or less.

References

Ashford, A., Barclay, W.R., Weaver, C.A., Giddings, T. H. and Zeller, S. (2000) Electron microscopy may reveal structure of docosahexaenoic acid-rich oil within *Schizochytrium sp. Lipids*, 35:1377–1386.

Chaney, A.L. and Marbatch, E.P. (1962) Modified reagents for determination of urea and ammonia *Clin Chem*, 8:130–135.

Christie, W.W., 2003. Lipid analysis. Pergamon, Oxford, 3^{th} edition, 277 pp.

Fernández-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M. and Montero, D. (1997) The effect of dietary protein and lipid from squid and fish meals on the egg quality of broodstock for gilhead seabream (*Sparus aurata*). *Aquaculture*, 148:233–246.

Folch, J., Lees, M. and Stanley, G.H.S. (1956) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*, 226(1):497–509.

Kanazawa, A., Koshio, S. and Teshima, S. (1989) Growth and survival of larval red seabream *Pagrus major* and japanese flounder *Paralichthys olivaceus* fed microbound diets. *J World Aquacult Soc*, 20:31–37.

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, J., Vergara, J.M., Montero, D. and Fernández-Palacios, H. (1995) Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. *Aquaculture*, 130:219–233.

Rodríguez-Ruiz, J., Belarbi, E.H., Sánchez, J.L.G. and Alonso, D.L. (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotech Tec*, 12(9):689–691.

Teshima, S., Kanazawa, A. and Sakamoto, S. (1982) Microparticulate diets for the larvae of aquatic animals. *Data File Fish Res Kagoshima Univ*, 2:67–86.

Chapter 3

Lipid accumulation in *Schizochytrium* G13/2S produced in continuous culture

This work was made at the University of Hull (England) and published in: Ganuza, E. and Izquierdo, M.S. (2007) *Appl Microbiol Biotech*, 270(1–4):178–185.

Abstract

Lipid and docosahexaenoic acid (DHA) accumulation in *Schizochytrium* G13/2S was studied under batch and continuous culture. Different glucose and glutamate concentrations were supplemented in a defined medium. During batch cultivation, lipid accumulation, 35 % total fatty acids (TFA), occurred at the arithmetic growth phase, but ceased when cell growth stopped. When continuous culture was performed under different glutamate concentrations, nitrogen-growth-limiting conditions induced the accumulation of 28–30 % TFA in *Schizochytrium*. As the dilution rate decreased from 0.08 to 0.02 h^{-1}, both cell dry weight and TFA content of the cell increased. Under a constant dilution rate of 0.04 h^{-1}, carbon-limiting conditions decreased the TFA to 22 %. Fatty acid profile was not affected by the different nutrient concentrations provided during continuous culture. Consequently, lipid accumulation can be induced through the carbon and nitrogen source concentration in the medium in order to maximize the TFA and subsequently DHA productivity by this microorganism.

Introduction

Schizochytrium sp. is a marine thraustochytrid that synthesises polyunsaturated fatty acids in which the valuable dietary docosahexaenoic acid (DHA, 22:6 n-3) represents 30-40 % of the total fatty acids (TFA) (Yokochi *et al.*, 1998). DHA is an essential polyunsaturated fatty acid for most vertebrates, which in humans is involved in the prevention of several diseases (Horrocks and Yeo, 1999) including cardiovascular (Kris-Etherton *et al.*, 2002) and neurological (Cole *et al.*, 2005) related diseases. For this reason, this

organism is receiving much attention as a source of enrichment in the feeds for aquaculture, laying hens and dairy cows (Barclay *et al.*, 2005).

The optimization of the oil production process requires the study of the lipid accumulation biochemistry and the definition of the environmental and nutritional factors affecting lipid quantity and quality. The capacity to accumulate lipids in oleaginous microorganisms is usually associated to some nutrient limiting conditions such as nitrogen (Gill *et al.*, 1977; Granger *et al.*, 1993; Ratledge, 2002). At present, *Schizochytrium* sp. is produced in batch cultures where TFA is at least 45 % cell dry weight (CDW) (Yaguchi *et al.*, 1997), however the studies on lipid accumulation processes and factors to enhance DHA production are scarce. Actually, during a batch cultivation, the nutrient concentration of the medium and the growth rate of the cultured organism vary during fermentation, which makes difficult to comprehend the process. In contrast, in continuous culture, under chemostat conditions, all these parameters remain constant and it is possible to isolate a single parameter and to study its effect on lipid accumulation while maintaining other physical and chemical parameters constant. Nevertheless, there are no publications on the continuous culture of this organism.

Lipid accumulation in fungi has been well studied under continuous culture. For instance, the oleaginous fungus *Candida* 107 attains a maximum oil content in nitrogen-limited chemostat cultures with dilution rates of about one-third of the maximum specific growth rate (μ_{max}) (Gill *et al.*, 1977), whereas carbon-limitation and high dilution rates are usually detrimental for lipid accumulation in this organism (Hall and Ratledge, 1977). Therefore, the oil content and fatty acid profile in fungi could be modified by factors such as dilution rate and growth-limiting nutrients among others. The objective of the present study was to determine the lipid accumulation processes in *Schizochytrium* G13/2S cultured in batch and continuous systems, under different carbon or nitrogen restrictions and at different dilution rates.

Materials and methods

Chemicals Glutamic acid monosodium salt was obtained from BDH (Poole, England); Thiamine, biotin, cyanocobalamin and nonadecanoic acid were obtained from Sigma; Proteose peptone and yeast extract were supplied by Oxoid; Polypropylene glycol-2000, was obtained from Fisher Scientific (Loughborough, England).

Organism and culture conditions *Schizochytrium* sp. strain G13/2S, derived from G13 (Graham Bremer, University of Portsmouth) and deposited at Banco Nacional de Algas (BNA 40–004) (Taliarte, Spain), was preserved

(2–3 months) in screw-capped tubes (10 ml) at 10 °C before inoculating (10 % v/v) the starting cultures. Sub-culturing of the strain was made in static flask of 50 ml (containing 10 ml medium) followed by 250 ml flask (50 ml). The initial medium contained ($g\ l^{-1}$): sea salts (27), glucose (40), proteose peptone (8), yeast extract (5) and MOPS (21). Further cultures were grown in a defined medium modified from Ashford *et al.* (2000) containing ($g\ l^{-1}$): glucose (40), NaCl (12.5), glutamic acid monosodium salt (4), $MgSO_4\ 7H_2O$ (2.5), KCl (0,5), $CaCl_2$ (0.1), KH_2PO_4 (0.5), unless stated otherwise. This medium also contained trace-element solution (5 $ml\ l^{-1}$) and vitamin solution (1 $ml\ l^{-1}$). The trace element solution contained ($g\ l^{-1}$): EDTA di-sodium salt (6.0), $FeCl_3\ 6H_2O$ (0.29), H_2BO_3 (6.84), $MnCl_2\ 4H_2O$ (0.86), $ZnCl_2$ (0.06), $CoCl_2\ 6H_2O$ (0.026), $NiSO_4\ 6H_2O$ (0.052), $CuSO_4\ 5H_2O$ (0.002), $Na_2MoO_4\ 2H_2O$ (0.005). The vitamin solution contained ($mg\ l^{-1}$): thiamine (100), biotin (0.5) and cyanocobalamin (0.5). Glucose was autoclaved separately. The feeding medium for continuous culture was filter-sterilized (pore size, 0.2 μm).

Static-flask cultures (3–7 d, 27 °C) were used to inoculate (10 % v/v) shake-flask cultures (250 ml, 120 rpm, 27 °C) containing 50 ml of defined medium. The medium used in flask cultures was buffered with 1 M MOPS and pH was adjusted to 7 by addition of KOH before autoclaving (121 °C, 15 min). Shake-flask cultures (24 h) were used to inoculate the fermenters (10 % v/v).

Batch and continuous cultures were performed in in-house manufactured (stainless steel and glass) fermenter with a total volume of 2.5 l, equipped with a vaned disk impeller, containing 2 l medium. In order to obtain dissolved O_2 concentration above 50 % throughout the whole fermentation, vortex mixing was provided with a vaned-disk impeller stirred at 1000 rpm while aeration was set to 0.5 vol. air (vol. medium)$^{-1}$ min^{-1}. Foaming was controlled in batch culture by manual addition of polypropylene glycol-2000 when necessary. Addition of antifoam was not needed during continuous culture. In fermenter cultures, the temperature was set to 27 °C and pH maintained at 7 with automatic addition of H_2SO_4 (2 M) or KOH (2 M). During continuous culture, the volume in the vessel was maintained using an overflow weir. Medium was supplied (24 h after inoculation) with a flow inducer at a constant dilution rate (D). Steady-state conditions were maintained for more than five complete volume changes before sampling (< 100 ml), while dissolved O_2 output was used to detect any perturbation (> 3 % saturation). Different dilution rates were applied starting from low and going to higher values in order to permit the cells adaptation. Under these experimental conditions, a single replicate (n=1) of the following fermentations was performed: batch cultivation of *Schizochytrium* G13/2S with 40 g glucose l^{-1},

continuous culture at different glutamic acid monosodium concentrations (6 to 4 $g\ l^{-1}$), batch cultivation in the absence of glucose, continuous culture at different glucose concentrations (7 to 40 $g\ l^{-1}$) and finally continuous culture with different medium dilution rates (0.08 to 0.02 h^{-1}).

Determination of cell dry weight The culture samples (20–100 ml) where harvested by centrifugation (3000 g, 5 min, 10 °C) and biomass was washed twice in demineralised water. The pellet fraction was freeze-dried and dry biomass was then measured.

Lipid analysis Fatty acid methyl esters (FAMEs) were prepared by the method described by Rodríguez-Ruiz *et al.* (1998) with nonadecanoic acid as internal standard. Fatty acid methyl esters were separated by GC with He as a carrier gas using a BPX70 25 m x 0.22 mm i.d. column (SGE). The temperature of the column was raised from 160 °C to 225 °C at 5 °C min^{-1} and then maintained at 225 °C for further 5 min. Peak detection was performed by a flame ionisation detector. The temperature of the injection port and flame-ionisation port was 250 °C. Fatty acids were identified by comparison to well characterised external standards (Sigma).

Determination of nitrogen content The supernatant and cell biomass were freeze-dried in each sample and 10 mg was then analyzed by a quantitative dynamic flash combustion method for elementary C, H, N and S detection. Instantaneous oxidation was attained in a 1020 °C combustion reactor supplied with O_2. Excess of O_2 was then reduced through a Cu reduction catalyst layer. The elements were separated in a 2 m chromatographic column packed with Porapak QS of 80/50 mesh size and ran at 60 °C using He as carrier gas. Detection was achieved by thermal conductivity.

Glucose determination The residual glucose concentrations were analysed using the GOD-PERID method (Boehringer Mannheim). The reagent was prepared every two weeks using horseradish peroxidase (0.8 units ml^{-1}), ABTS (1 mg ml^{-1}) and glucose oxidase (10 units ml^{-1}) diluted in a 100 mM phosphate buffer (pH 7). The reactive was always compared with the internal standard provided with the test kit.

Results

Lipid accumulation in batch culture In batch cultivation of *Schizochytrium* G13/2S with glucose (40 $g\ l^{-1}$) and glutamate (4 $g\ l^{-1}$), lipid pro-

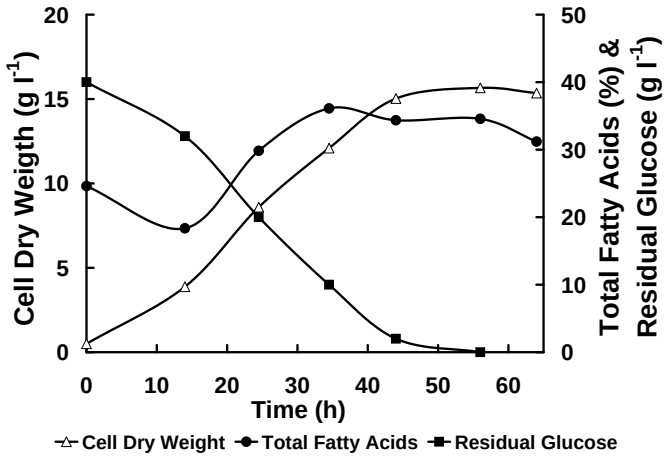


Figure 3.1: Growth, total fatty acid (% of CDW) and residual glucose of *Schizochytrium* G13/2S batch-cultured in a medium containing 40 $g\ l^{-1}$ glucose and 4 $g\ l^{-1}$ glutamic acid monosodium. The curve represents typical values of multiple fermentations carried out under similar conditions.

duction, expressed in terms of TFA, markedly increased during the late arithmetic growth phase, reaching about 35 % of CDW, and being kept constant for the rest of the culture, until residual glucose was completely depleted (Figure 3.1). At the stationary phase, the proportions of the main fatty acids (% of TFA) were: myristic acid (14:0) (5–7 %), palmitic acid (16:0) (32–33 %), stearic acid (18:0) (2 %), docosapentaenoic acid (22:5 n-6) (10–12 %) and DHA (22:6 n-3) (43–47 %).

Effect of nitrogen source concentration in continuous culture The biomass of the fermenter increased with an elevation of glutamic acid monosodium concentration up to 4–6 $g\ l^{-1}$, whereas TFA accumulation was highest (28–30 %) at the lowest glutamate concentrations (2–4 $g\ l^{-1}$) (Table 3.1). Further increase of glutamate to 6 $g\ l^{-1}$ markedly decreased TFA production, indicating a higher lipid accumulation in the cell when nitrogen source is limiting growth. Regardless of the initial glutamate concentration, all residual nitrogen was absorbed by the cell as indicated by the elementary analysis of the residual medium. Excess nitrogen concentration was used for biomass production (4 g glutamate l^{-1}) but also stored into the cell (6 $gglutamatel^{-1}$), doubling the nitrogen content in CDW. Glucose consumption correlated with the amount of biomass produced. Fatty acids profiles were not affected by the glutamate concentration and DHA represented a

Table 3.1: Effect of glutamic acid concentration on growth and lipid accumulation of *Schizochytrium* G13/2S at a constant dilution rate of 0.04 h^{-1}.

	Glutamic acid monosodium salt ($g\ l^{-1}$)[a]		
	2	4	6
Initial glucose ($g\ l^{-1}$)	40	40	40
Residual glucose ($g\ l^{-1}$)	22	20	20
Initial nitrogen (% w/w)[b]	0.4	0.5	0.8
Residual nitrogen (% w/w)[b]	0	0	0
Cell dry weight (CDW) ($g\ l^{-1}$)	6.2	8	7.8
Nitrogen in CDW (% w/w)[b]	1.9	2.5	4.6
Total fatty acids (% w/w of CDW)	28	30	19
Fatty acyl residues (% w/w of TFA)[c]			
14:0	4	4	3
16:0	39	37	39
18:0	3	3	3
22:5 n–6	10	10	9
22:6 n–3	41	41	41
Minor fatty acids	3	5	5

[a] The experiment was performed once while each sample was analyzed in triplicates. The values were quoted in order to include the analytical error.

[b] Nitrogen was detected by elementary analysis, detection limit was 0.01 %. Nitrogen values are represented as % w/w of C, H, N, S present in the freeze-dried and supernatant respectively.

[c] In all samples small amounts of 17:0, 15:0, $<$ 2 % total fatty acids (TFA), were detected.

constant percentage (41 % of TFA).

Carbon source limitation in batch culture When glucose was not provided in the media cell growth was limited and the storage lipids, the residual glucose from inoculum and the glutamate provided (4 g CDW l^{-1}) were only sufficient to produce growth of 2.5 g CDW l^{-1} in the first 20 h of culture (Figure 3.2). Hence, during this period lipids were consumed and the TFA content of the cell decreased down to 10 % of CDW. When growth ceased, the proportions of the main fatty acids were (% of TFA): palmitic acid (16:0) (24), docosapentaenoic acid (22:5 n-6) (13) and DHA (22:6 n-3) (54), indicating that polyunsaturated fatty acids were preferentially conserved by the

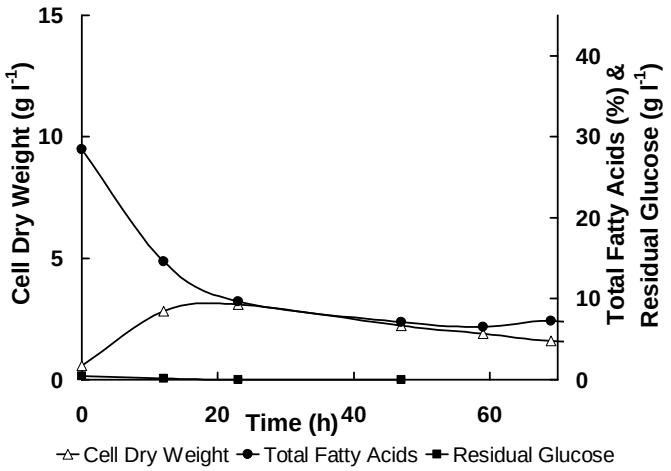


Figure 3.2: Growth, total fatty acid (% of CDW) and residual glucose of *Schizochytrium* G13/2S batch-cultured in a carbon source-limited medium. The cells were fed on glutamic acid monosodium (4 $g\ l^{-1}$) and the residual substrate remaining from the inoculum (1 $g\ l^{-1}$) as sole carbon sources. The curve represents typical values of multiple fermentations carried out under similar conditions.

cell.

Effect of carbon source concentration in continuous culture Both CDW and TFA production were increased by the elevation of glucose concentration in the medium up to 14 $g\ l^{-1}$, whereas further elevation of glucose did not improved lipid accumulation and only slightly CDW (Table 3.2). Nevertheless, TFA production was still high even at the lowest glucose concentration (22 % of CDW). In fact, lipid bodies were observed at phase-contrast microscopy (400 X) inside the cells. Proportions of the different fatty acids were not altered by glucose concentration, except for a small fluctuations. Thus, DHA and palmitic acid were the main fatty acids and their production remained approximately constant regardless of the glucose concentration assayed.

Effect of dilution rate in continuous culture Chemostat culture of *Schizochytrium* G13/2S was conducted using a nitrogen limited medium (2 g glutamate l^{-1} and 40 g glucose l^{-1}) under dilution rates ranging from 0.08 to 0.02 h^{-1}. CDW and TFA increased linearly with the decreasing dilution rate (Figure 3.3). Highest biomass (7.7-6.2 g CDW l^{-1}) and lipid accumulation (31-28 % TFA) was obtained at the lowest dilution rates assayed (0.02

Table 3.2: Effect of glucose concentration on growth and lipid accumulation of *Schizochytrium* G13/2S at a constant dilution rate of 0.04 h^{-1}.

	Glucose ($g\ l^{-1}$)[a]		
	7	15	40
Initial glucose ($g\ l^{-1}$)[b]	6	14	41
Residual glucose ($g\ l^{-1}$)	0	0	19
Cell dry weight ($g\ l^{-1}$)	1.8	5.1	5.5
Total fatty acids (% w/w of CDW)	22	28	28
Fatty acyl residues (% w/w of TFA)			
14:0	4	4	3
16:0	39	37	39
18:0	3	3	3
22:5 n–6	8	12	9
22:6 n–3	41	39	40
Minor fatty acids	5	5	6

[a] Medium contained $(NH_4)_2SO_4$ (0.706 $g\ l^{-1}$) instead of glutamic acid with the aim of providing glucose as the only carbon source. Analyses carried out as given in Table 3.1.

[b] The small deviation (< 10 %) observed between the projected glucose concentrations and the glucose analysed were derived from the handling of large volumes during medium preparation.

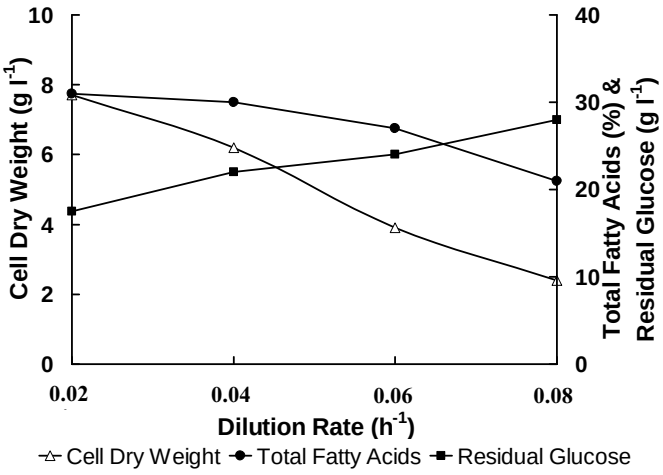


Figure 3.3: Dilution rate on the cell dry weight, total fatty acid (% of CDW) and residual glucose of *Schyzochytrium* G13/2S grown in a medium containing 40 g glucose l^{-1} and 2 g glutamic acid monosodium salt l^{-1}.

and 0.04 h^{-1} respectively). Accordingly, in both carbon and nitrogen source experiments, a 0.04 h^{-1} dilution rate was used in order to generate the maximum DHA productivity (r_{DHA}), which was 39 mg DHA l^{-1} h^{-1}.

Discussion

Nutrient limitation, usually nitrogen, is the key-factor inducing lipid accumulation in oleaginous microorganisms (Gill *et al.*, 1977; Ratledge and Wynn, 2002). When non-lipidic growth ceases in a batch culture, lipids are accumulated during the subsequent lipogenic phase. However, in the present experiment lipids were synthesised by *Schizochytrium* G13/2S during growth phase, as it could be observed when the organism was batch-cultured with glucose, suggesting that the pattern of lipid accumulation differs from that studied in other oleaginous organisms (Ratledge, 2002). This particular pattern was also observed in *Cryptococcus terricolus* (Boulton and Ratledge, 1984) and *Mortierella alpina* LPM301 (Eroshin *et al.*, 2002). As demonstrated under continuous culture conditions, *Schizochytrium* G13/2S assimilates and also accumulates the nitrogen sources, such as glutamate, from the medium. This capacity to store intracellular nutrient supplies would allow cell proliferation once the limited nutrient is depleted from the medium, permitting simultaneous growth and lipid synthesis. Beside this particular pattern of lipid formation, nitrogen source still plays an important role in inducing lipid accumulation in *Schizochytrium*. Under continuous cul-

ture, this growth-limiting nutrient was targeted and allowed for the growth rate to be isolated from other variables. Cell growth in the chemostat continuous culture was clearly related to nitrogen source concentration in the feeding medium and denoted that a concentration of (2 g glutamate l^{-1}) was growth-limiting. The increase in cell fatty acid content, with a constant DHA proportion, under nitrogen-growth limiting conditions showed that nitrogen source limitation is a key parameter inducing lipid accumulation also in *Schizochytrium* G13/2S. Thus, DHA production could be modulated by controlling the nitrogen source concentration.

In the absence of a carbon source, oleaginous microorganisms use their storage lipids for growth or cell maintenance. For instance, *Candida* 106 maximum lipid content under carbon-limited chemostat culture was 14 % of CDW (Gill *et al.*, 1977). Under a batch culture, *Schizochytrium* G13/2S was able to consume the storage lipids and grew even when TFA was only 10 % of CDW, suggesting that this is the constitutive minimum amount required for growth. However, under carbon limited chemostat culture, TFA turn out to be 22 %. This percentage must does not represent only structural lipids, given that oil droplets were visible at the microscope. Hence, lipid accumulation in *Schizochytrium* G13/2S grown in carbon-limited chemostat culture seems to be a peculiarity of this microorganism, which has not been described in other oleaginous species.

The degree of unsaturation and fatty acid profile in oleaginous microorganisms could be manipulated during continuous culture by several factors such as the amount of carbon and nitrogen sources (Gill *et al.*, 1977), different carbon sources and dilution rates (Evans and Ratledge, 1983), dissolved O_2 (Roux *et al.*, 1995) or temperature (Kendrick and Ratledge, 1992). However, the fatty acid profile of *Schizochytrium* G13/2S shows a strong dominance of palmitic acid, DPA and DHA under all the conditions studied (Tables 3.1–3.2), which indicates that physiological-tailoring of the fatty acids profile in order to increase DHA proportion might be more complicated than in other microorganisms. Nevertheless, it has also been demonstrated that nitrogen and carbon sources concentrations in the medium can be adjusted in order to induce lipid synthesis during the growth phase of *Schizochytrium* G13/2S, permitting an optimum DHA accumulation.

Table 3.3: Comparison of growth, biomass productivity (r_{CDW}), DHA productivity (r_{DHA}), specific rate of DHA formation (μ_{DHA}), biomass yields and total fatty acid (TFA) accumulation of *Schizochytrium* G13/2S under batch and continuous cultivation.

Culture configuration[a]	Batch		Continuous
Time (h)	44	56	Steady state
Cell dry weight (CDW) ($g\ l^{-1}$)	15	15.7	8
r_{CDW} ($g\ l^{-1}\ h^{-1}$)	0.33	0.27	0.32
r_{DHA} (mg DHA $l^{-1}\ h^{-1}$)	50	46	40
μ_{DHA} (mg DHA g CDW$^{-1}\ h^{-1}$)	3.4	2.9	4.9
Yield (g CDW g^{-1} glucose)	0.39		0.39
TFA (% w/w of CDW)	35	35	30
Fatty acyl residues (% w/w of TFA)			
14:0	7	5	4
16:0	33	32	37
18:0	2	2	3
22:5 n–6	12	10	10
22:6 n–3	43	47	41
Minor fatty acids	3	4	5

[a] Media for batch and continuous culture contained glucose (40 $g\ l^{-1}$) and glutamic acid monosodium salt (2 $g\ l^{-1}$). Batch culture inoculum was 10 % v/v and continuous culture dilution rate was 0.04 h^{-1}. Analyses carried out as given in Table 3.1.

Production of lipids and other secondary metabolites is usually a bi-phasic process and batch cultures are preferred for industrial production. However, within growth-associated lipid accumulating microorganisms, single-stage continuous cultures may prove suitable for lipid and DHA production. Under the range of dilution rates tested, 0.04 h^{-1} gave the best r_{DHA}. Detailed comparison between batch and continuous culture (D=0.04 h^{-1}) grown in the same medium highlights interesting information (Table 11.2). Despite batch r_{DHA} (50-46 mg DHA l^{-1} h^{-1}) seeming superior to continuously cultured *Schizochytrium* G13/2S r_{DHA} (39 mg DHA l^{-1} h^{-1}), recalculation in batch cultures, considering a "turn around" time of 24 h necessary to set up the new batch fermentation, gives a final value of 32 mg DHA l^{-1} h^{-1} compared to the 39 mg DHA l^{-1} h^{-1} attained in continuous cultures. Moreover, the harvesting method in continuous culture (e.g, continuous centrifugation) is far more efficient than the single large volume that needs to be harvested under batch cultivation. Since biomass yields in glucose were similar during both culture configuration and glucose could be adjusted in the feeding medium to the organisms requirements, the r_{DHA} improvement will suppose an economic advantage to the fermentation process, thus highlighting the potential of continuous culture in *Schizochytrium* fermentation under optimum dilution rates.

Acknowledgements

This work was supported by Tomás de Zárate Grant (Cabildo de Gran Canaria). We thank Colin Ratledge, Alistair Anderson and Simon Hopkins for his help and assistance in this work.

References

Ashford, A., Barclay, W.R., Weaver, C.A., Giddings, T. H. and Zeller, S. (2000) Electron microscopy may reveal structure of docosahexaenoic acid-rich oil within *Schizochytrium* sp. *Lipids*, 35:1377–1386.

Barclay, W., Weaver, C. and Metz, J. (2005) Development of a docosahexaenoic acid production technology using *Schizochytrium*: a historical perspective. In: Ratledge, C. and Cohen, Z. (Eds.), Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 36–49.

Boulton, C.A. and Ratledge, C. (1984) *Cryptococcus terricolus*, an oleaginous yeast re-appraised. *Appl Microbiol Biotech*, 20(1):72–76.

Cole, G. M., Lim, G. P., Yang, F., Teter, B., Begum, A., Ma, Q., Harris-

White, M. E. and Frautschy, S. A. (2005) Prevention of Alzheimer's disease: Ω-3 fatty acid and phenolic anti-oxidant interventions. *Neurobiol Aging*, 26(1):133–136.

Eroshin, V.K., Dedyukhina, E.G., Satroutdinov, A.D. and Chistyakova, T.I. (2002) Growth-coupled lipid synthesis in *Mortierella alpina* LPM301, a producer of arachidonic acid. *Microbiology*, 71(2):169–172.

Evans, C.T. and Ratledge, C. (1983) A comparison of the oleaginous yeast, *Candida curvata*, grown on different carbon-sources in continuous and batch culture. *Lipids*, 18(9):623–629.

Gill, C.O., Hall, M.J. and Ratledge, C. (1977) Lipid-accumulation in an oleaginous yeast (*Candida* 107) growing on glucose in single-stage continuous culture. *Appl Environ Microbiol*, 33(2):231–239.

Granger, L.M., Perlot, P., Goma, G., and Pareilleux, A. (1993) Effect of various nutrient limitations on fatty-acid production by *Rhodotorula glutinis*. *Appl Microbiol Biotech*, 38(6):784–789.

Hall, M.J. and Ratledge, C. (1977) Lipid-accumulation in an oleaginous yeast (*Candida* 107) growing on glucose under various conditions in a one-stage and two-stage continuous culture. *Appl Environ Microbiol*, 33(6):577–584.

Horrocks, L.A. and Yeo, Y.K. (1999) Health benefits of docosahexaenoic acid (DHA). *Pharmacol Res*, 40(3):211–225.

Kendrick, A. and Ratledge, C. (1992) Lipid formation in the oleaginous mold *Entomophthora exitalis* grown in continuous culture. Effects of growth-rate, temperature and dissolved-oxygen tension on polyunsaturated fatty-acids. *Appl Microbiol Biotech*, 37(1):18–22.

Kris-Etherton, P.M., Harris, W.S. and Appel, L.J. (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*, 106(1):2747–2757.

Ratledge, C. (2002) Regulation of lipid accumulation in oleaginous microorganisms. *Biochel Soc T*, 30:1047–1050.

Ratledge, C. and Wynn, J.P. (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbiol*, 51:1–51.

Rodríguez-Ruiz, J., Belarbi, E.H., Sánchez, J.L.G. and Alonso, D.L. (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotech Tec*, 12(9):689–691.

Roux, M.P., Kock, J.L.F., du Preez, J.C. and Botha, A. (1995) The influence of dissolved oxygen tension on the production of cocoa butter equivalents and gamma-linolenic acid by *Mucor circinelloides*. *Syst Appl Microbiol*, 18(3):329–334.

Yaguchi, T., Tanaka, S., Yokochi, T., Nakahara, T. and Higashihara, T. (1997) Production of high yields of docosahexaenoic acid by *Schizochytrium* sp. strain SR21. *J Am Oil Chem Soc*, 74:1431–1434.

Yokochi, T., Honda, D., Higashihara, T. and Nakahara, T. (1998) Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. *Appl Microbiol Biotech*, 29:72–76.

Chapter 4

The influence of air supply on fatty acid production by *Schizochytrium* G13/2S and *Crypthecodinium cohnii*

This work, which remains un-published, was carried out at the fermentation laboratories of the University of Hull (England).

Abstract

Total fatty acid (TFA) and docosahexaenoic acid (DHA; 22:6 n-3) synthesis in the marine microheterotrop *Schizochytrium* G13/2S and *Crypthecodinium cohnii* were studied under different aerating conditions. Growth and TFA accumulation in shake-flask cultures gradually declined with decreasing air supply in both organisms. In contrast, the polyunsaturated fatty acid proportion, in particular DHA (around 40 % TFA), remained constant. Only oleic acid in *Crypthecodinium cohnii* declined from 12 to 7 % TFA with decreasing air supply. In addition, *Schizochytrium* G13/2S was batch-cultured in an O_2-free fermenter, purged with N_2 every two days. At the end of fermentation (37 d), 0.61 g cell dry weight l^{-1} containing 33 and 6.3 $mg\ l^{-1}$ of newly synthesised DHA and docosapentaenoic acid (22:5 n-6; DPA) respectively were produced. The stability of DHA in the fatty acid profile during air supply limitation in both organisms suggests that DHA desaturation might not be O_2-dependent as traditionally assumed.

Introduction

Docosahexaenoic acid (DHA; 22:6 n-3) is a polyunsaturated fatty acid present in human brain (Yavin *et al.*, 2002), retina (Jeffrey *et al.*, 2001) and heart tissue (Grynberg, 2003). *De novo* synthesis of this fatty acid is limited to a small number of organisms such as marine phytoplankton. Dietary terrestrial vegetables only produce DHA precursors like α-linolenic acid (ALA; 18:3 n-3) whose conversion to highly unsaturated fatty acids could be insufficient in humans (Gerster, 1998). Consequently, DHA must

be provided in diet as it plays a vital role in many aspects of human health (Wang *et al.*, 2003; Muskiet *et al.*, 2004).

The marine microalgae *Schizochytrium* sp. (Thraustochytrid) and *Crypthecodinium cohnii* (Dinoflagellate) are commercially cultivated to produce the valuable dietary DHA, which is included as a nutraceutical ingredient in diet (Cohen and Ratledge, 2005). Due to their heterotrophic growth, these algae can be cultivated in 100 m^3 fermenters where they can accumulate lipids to over 40 % of the cell dry weight (CDW) containing DHA around 35 % of total fatty acids (TFA). Since large-volume industrial scale fermentation is usually restricted by O_2 availability in the culture, it is particularly interesting to investigate whether the deficient aeration could affect the DHA content of these organisms and compromise their productivities.

The fatty acid response to air supply might also provide information about the biochemical pathway leading to the synthesis of polyunsaturated fatty acids. In *Schizochytrium*, a novel pathway based on the non-O_2 dependent polyketide synthase (PKS) enzymatic complex has been proposed (Metz *et al.*, 2001). This pathway does not employ O_2-dependent desaturases like those of the traditional pathways and should not, therefore, compromise DHA production under the low O_2 levels often attained in large-scale fermentation. *C. cohnii*, as well as *Schizochytrium* sp., possess no intermediate fatty acid products from this traditional elongase-desaturase series in their fatty acid profile. Attempts to detect desaturase activity in *C. cohnii*, incubating with their inhibitors and radiolabelled fatty acyl precursors, as well as to identify active desaturases have failed so far (de Swaaf *et al.*, 2003; Ratledge, 2004). Consequently, the pathway for DHA synthesis is still unclear in these organisms. Studying the effect of aeration on DHA production may contribute to clarify the DHA synthesis route.

The aim of the present study was to determine whether *Schizochytrium* G13/2S and *C. cohnii* synthesise DHA through a pathway that requires O_2 and how this might interfere with the commercial bioprocess design.

Materials and Methods

Chemicals Proteose peptone and yeast extract were obtained from Oxoid. Sea salts and heptadecanoic acid and nonadecanoic acid were supplied by Sigma; Polypropylene glycol-2000 grade was obtained from Fisher Scientific (Loughborough, England).

Organism and culture conditions *Schizochytrium* sp. strain G13/2S derived from G13 (Graham Bremer, University of Portsmouth) was grown in a medium containing ($g\ l^{-1}$): sea salts (27), glucose (40-80), proteose peptone

(8), yeast extract (5) and MOPS (21); pH was adjusted to 7 ±0.3 with KOH (1 M). *Crypthecodinium cohnii* ATCC 50060 was grown in a medium containing ($g\ l^{-1}$): sea salts (25), glucose (27) and yeast extract (2); pH was adjusted to 6.5 ±0.3 with NaOH (1 M). Both organisms were grown in shake flasks (120 rpm, 250 *ml*) at 27 °C. Air availability was tested in two different ways in order to exclude the effect of hydrodynamics in the flask: (1) under different culture medium surface to medium volume ratio using 50, 150 and 250 *ml* of medium; (2) with 50 *ml* of medium either normally aerated or sealed with craft paper in a previously N_2-gassed atmosphere.

Fermenter cultures were grwon in a laboratory bioreactor (Braun Biolab) with a working capacity of 1 *l*. Foam production was controlled by addition of polypropylene glycol-2000 when necessary and pH 7 was maintained by automatic addition of 2 M KOH or H_2SO_4 as required. A stirred with Rushton blades was used and set to 500 rpm. Two fermentations were performed simultaneously using both anaerobic and aerobic fermenters. The anaerobic fermenter was leak tested under 5 psi of positive pressure before inoculation and after cultivation. In order to ensure anaerobic atmosphere, the culture was sparged with high-purity-filter-sterilised (99.99 %) N_2 (Engineering and Welding, Hull, UK) for ten minutes every two days and all the silicon-rubber tubing were clipped. The aerobic bioreactor was maintained within the same conditions, but with 0.5 vol. air income per vol. medium^{-1} per min^{-1}.

All cultures were inoculated at 10 % v/v with 50 *ml* shake-flask cultures of 1 *d* for *Schizochitrium* G13/2S and 3 *d* for *C. cohnii*. All components were autoclaved (120 °C) for 15 *min* maintaining glucose in separate containers.

Determination of cell dry weight Medium samples (20-100 *ml*) where harvested by centrifugation (3000 *g*, 5 min, 10 °C) and biomass was washed twice in demineralised water. The pellet fraction was freeze-dried and dry biomass was then measured.

Fatty acid analysis Fatty acid (FA) analysis of *C. cohnii* freeze-dried biomass (25–30 *mg*) was conducted using heptadecanoic acid (2.5 *mg* per sample), previously dissolved in methanol: chloroform 3:1, as an internal standard. *Schizochytrium* G13/2S biomass (10–15 *mg*) was analysed using nonadecaenoic acid (1.25 *mg* per sample), previously dissolved in hexane, as internal standard. Fatty acid methylation was conducted by the method of Rodríguez-Ruiz et al. (1998). Fatty acid methyl esters were then analyzed by GC using He as a carrier gas with a BPX70 25 *m* x 0.22 *mm* i.d. column (SGE). The temperature of the column was raised from 160 °C to 225 °C at 5 °C min^{-1} and then maintained at 225 °C for further 5 *min*. The tempera-

tures of the injection and detection ports were 250 °C. Peaks were identified by comparison with well characterised standards.

Glucose determination The residual glucose concentrations were analysed using the GOD-PERID method (Boehringer, Mannhein). The reagent was prepared every two weeks using horseradish peroxidase (0.8 $units\ ml^{-1}$), ABTS (1 $mg\ ml^{-1}$) and glucose oxidase (10 units ml^{-1}) diluted in a 100 mM phosphate buffer (pH 7). The reactive was always compared with the internal standard provided on the test kit.

Results

The effect of air supply in shake-flask cultures was observed in both type of trials, performed either with different medium-surface to volume ratio or with a constant working volume maintained with air or N_2-gassed atmosphere. Glucose utilization, CDW and TFA accumulation was poor when air supply was low in *Schizochytrium* G13/2S (Tables 4.1 and 4.2) and *C. cohnii* (Tables 4.3 and 4.4). CDW limitation by the air supply was higher in *Schizochytrium* G13/2S than in *C. cohnii* in accordance with the higher growth rate of this organisms. Both organisms presented a very stable fatty acid profile; DHA proportion in fatty acyl residues, around 40 % of TFA, was steady for all treatments. Only oleic acid (18:1 n-9, OA) in *C. cohnii* increased with the enhanced air supply from 7 to 12 % of TFA. Other fatty acids were not affected, or showed only small variations with by air supply.

Schizochytrium G13/2S was cultured for 37 d in an anaerobic fermenter. Zoospores were present in the N_2-sparged fermenter during the first 12 d. Dividing cells were present all along the fermentation period suggesting that culture was well adapted to anoxia. The first three days of culture cell biomass decreased but then it began to grow, showing a subsequent ten-fold increase (Figure 4.1). Overall glucose consumption was 17 $g\ l^{-1}$. The step increase in CDW and residual glucose, as well as the low evaporation (< 5 %) obtained at the end of anaerobic fermentation denoted an evident growth. Any O_2 that might permeate through the silicon tubing or the addition of acid or alkali was not reflected on the O_2 probe indicating that the scarce O_2 available in the fermenter was quickly consumed by the organisms, which further supported anaerobic conditions. Biomass final concentration was 0.8 g CDW l^{-1} while the TFA of the cells remained below 20 % of CDW. New DHA (32.9 mg) was synthesised as well as other unsaturated fatty acids. As expected, the control aerobic bioreactor showed better growth attaining 21.4 $g\ l^{-1}$ after 78 h of culture. No contamination was observed in any of the samples after detailed analysis at the microscope. At the end of the culture,

Table 4.1: Effect of air supply in *Schizochytrium* G13/2S shake-flask cultures under different medium-surface to volume ratio.

Time (h)	0	48		
Working volume	50	50	150	250
Residual glucose ($g\ l^{-1}$)	39	2	17	23
Cell dry weight ($g\ l^{-1}$)	0.5	8.6	4.9	1.6
Total fatty acid (% of CDW)	28	33	25	17
Fatty acyl residues (% w/w of TFA)				
14:0	4	4	4	2
15:0	6	3	10	7
16:0	42	38	31	33
17:0	2	1	3	3
18:0	2	2	1	1
22:5 n-6	8	10	10	11
22:6 n-3	35	41	40	42
Other FA	1	1	1	1

[a] Time 0 *h* sample was taken just after inoculation. Total fatty acids (TFA). The values represent the average of three different replicates cultured in 250 ml Erlenmeyer flask. All figures have been quoted in order to include the error of the replicates.

no leaks were observed under 5 *psi* at the anaerobic fermenter.

Discussion

During microbial cultures, O_2 limitation will constrain the recycling of metabolic intermediates (i.e. NADPH) in the electron-transport-coupled phosphorylation (Tsai *et al.*, 1995). The consequent metabolic down-regulation results in the culture attaining low growth, as observed in *Schizochytrium* G13/2S and *C. cohnii* experiments. The low biomass attained under O_2 limitation will decrease the nutrient utilization, thus preventing nitrogen-limiting conditions, which induces the accumulation of lipids within the cell (Ratledge, 2004; Ganuza and Izquierdo, 2007). Accordingly, the lipid content under O_2-limitation was always below 20 % TFA. Therefore, the little NADPH available in the cell appears to be preferentially consumed for the synthesis of protein, diverting NADPH utilisation, which is also required for lipid synthesis (Ratledge, 2004). The results obtained in the present study were in agreement with the decrease in *Candida* 107 lipid (Hall and Ratledge,

Table 4.2: Effect of air supply in *Schizochytrium* G13/2S shake-flask cultures under a modified N_2 atmosphere.

Time (h)	0	74	
Treatment	-	Aerobic	Anaerobic
Residual glucose ($g\ l^{-1}$)	58	0	39
Cell dry weight ($g\ l^{-1}$)	0.4	13.9	4.1
Total fatty acid (% of CDW)	26	29	15
Fatty acyl residues (% w/w of TFA)			
14:0	4	5	2
15:0	6	5	22
16:0	42	30	15
17:0	2	2	8
18:0	2	2	2
22:5 n-6	8	13	9
22:6 n-3	35	42	41
Other FA	1	1	1

[a] Experiment was performed as described in Table 4.1. Sterile N_2 was purged in the anaerobic flasks which were then covered with a cotton bung, parafilm and craft paper.

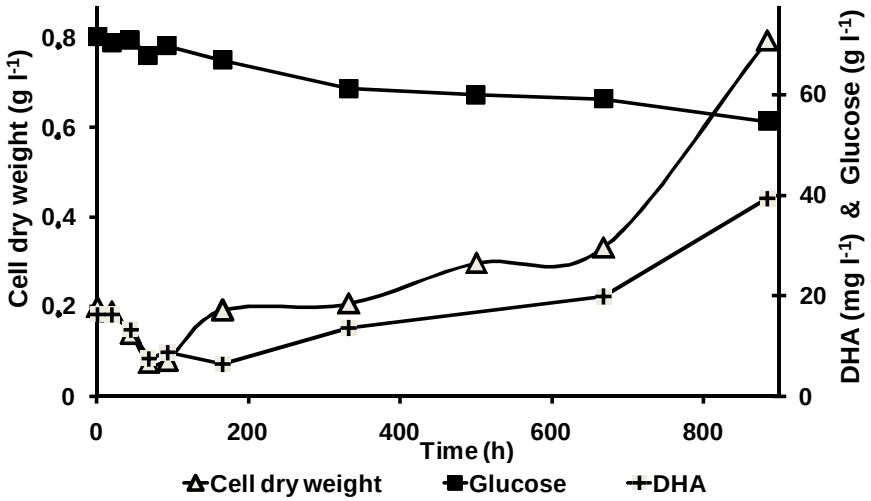


Figure 4.1: Biomass and DHA production, residual glucose consumption in *Schizochytrium* G13/2S, batch cultured in a fermenter under anaerobic conditions. The aerobic fermenter (not represented) was used as control and confirmed the viability of the shared inoculum.

Table 4.3: Effect of air supply in *Crypthecodinium cohnii* shake-flask cultures under different medium-surface to volume ratio.

Time (h)	0	48		
Working volume	50	50	150	250
Residual glucose ($g\ l^{-1}$)	26	0	13	16
Cell dry weight ($g\ l^{-1}$)	0.9	9.9	7.5	4.9
Total fatty acid (% of CDW)	15	19	11	10
Fatty acyl residues (% w/w of TFA)				
14:0	18	18	18	20
16:0	31	28	28	26
18:1	12	12	9	7
22:6 n-3	38	38	39	41
Other FA	1	4	6	6

[a] Experiment was performed as described in Table 4.1.

1977) and *Phaffia rhodozyma* astaxanthin content under low O_2 conditions (Yamane *et al.*, 1997). Although lipid accumulation in *C. cohnii*, strain GC, was inversely related to air supply (Beach and Holz, 1973), this was probably due to a two-day sampling difference applied between control and O_2 limited treatment employed in that experiment.

The synthesis of unsaturated fatty acids has been traditionally associated to the activity of fatty acid desaturases, which are O_2 dependent enzymes (Klein and Volkmann, 1975; Moreton, 1988). This condition is reflected on various yeasts that under poor O_2 conditions, decreases the unsaturation index of their fatty acids (Roux *et al.*, 1995; Valero *et al.*, 2001). In the present experiment polyunsaturated fatty acids were steady regardless of the different aerating conditions, suggesting that desaturase activity was not affected by O_2-limitation. The fatty acid desaturases are known to posses a very high affinity for O_2 (Meyer and Bolch, 1963; Klein and Volkmann, 1975) and might not be affected by O_2 growth-limiting conditions. However, the low O_2 conditions applied in *C. cohnii* were sufficient to decrease the production of OA, which appears to be related to the inhibition of Δ^9-desaturase activity. Accordingly, Lopes da Silva *et al.* (2006) found that, when O_2 transfer in *C. cohnii* CCMP316 fermentation was enhanced with n-dodecane, the relation between OA and DHA increased. Therefore, DHA and OA syntheses in *C. cohnii* appears to be differently affected by the air supply. Sonnenborn and Kunau (1982) observed that the fatty acid synthetase complex isolated in vitro from the cytosol of *C. cohnii* produced only saturated fatty acids, indicating that further desaturation to OA was probably made by a

Table 4.4: Effect of air supply in *Crypthecodinium cohnii* shake-flask cultures under a modified N_2-atmosphere.

Time (h)	0	72	
Treatment	-	Aerobic	Anaerobic
Residual glucose ($g\ l^{-1}$)	26	0	5
Cell dry weight ($g\ l^{-1}$)	0.6	9.8	8.4
Total fatty acid (% of CDW)	15	20	8
Fatty acyl residues (% w/w of TFA)			
14:0	18	18	20
16:0	31	28	29
18:1	12	12	8
22:6 n-3	38	38	37
Other FA	1	4	6

[a] Experiment was performed as described in Table 4.2.

membrane-bound desaturation. Besides, as it has been shown in radiolabeled experiments, the monounsaturated fatty acid is not a precursor of DHA, as the later is synthesised *de novo* by two-carbon-unit molecules rather than from fatty acids taken up from the medium (Beach *et al.*, 1974; Henderson and Mackinlay, 1991). Therefore, biosynthesis of DHA is compartmentalized from OA and, hence, it is possible that both fatty acids are differently affected by air supply in *C. cohnii*.

In the present experiment there was no variation in the proportion of DHA in either organisms in relation to the air supply. Moreover, *Schizochytrium* G13/2S appears to be capable of synthesising DHA and DPA in fermenter under the presence of very little, if any, O_2. The results from anaerobic fermentation were in good agreement with the new O_2-independent pathway (PKS) that apparently is used by *Schizochytrium* and requires less NADPH (Metz *et al.*, 2001). The operation of a bacterial-like PKS system, also in *C. cohnii*, would help to explain why no intermediate chain-length fatty acyl groups to DHA in their lipids were found and why it synthesises the uncommon octacosaoctaenoic acid (28:8n3). This uncommon fatty acids is also present in some thraustochytrids (Van Pelt *et al.*, 1999), only when the temperature program of the GC is further extended. In agreement with the action of PKS in *C. cohnii*, it was found that DHA proportions in fatty acyl residues were not altered by Δ^4, Δ^6 (de Swaaf *et al.*, 2003) and Δ^{15} desaturase inhibitors (Henderson *et al.*, 1990). We could now add to these evidences that desaturation of DHA was not affected by the O_2 supply, thus supporting the PKS pathway in both *Schizochytrium* sp. and *C. cohnii*. From a fermentation point of view, this finding ensures that the DHA pro-

portion in fatty acids is not directly endangered by the low O_2 attainable at some point of the large-scale fermentation.

Acknowledgements

This work was supported by Tomás de Zárate Grant (Cabildo de Gran Canaria). We thank Colin Ratledge, Alistair Anderson and Simon Hopkins for his help and assistance in this work.

References

Beach, D.H. and Holz, G.G., Jr. (1973) Environmental influences on the docosahexaenoate content of the triacylglycerols and phosphatidylcholine of a heterotrophic, marine dinoflagellate, *Crypthecodinium cohnii*. *Biochim Biophys Acta*, 316:56–65.

Beach, D.H., Harrington, G.W., Gellerman, J.L., Schlenk, H. and George G. Holz, J. (1974) Biosynthesis of oleic acid and docosahexaenoic acid by a heterotrophic marine dinoflagellate *Crypthecodinium cohnii*. *Biochim Biophys Acta*, 369:16–24.

Cohen, Z. and Ratledge, C. (2005) Single cell oils. *AOCS Press*, Champaign, Illinois, pp. 257.

de Swaaf, M.E., de Rijk, T.C., van der Meer, P., Eggink, G. and Sijtsma, L. (2003) Analysis of docosahexaenoic acid biosynthesis in *Crypthecodinium cohnii* by C-13 labelling and desaturase inhibitor experiments. *J Biotech,* 103:21–29.

Ganuza, E. and Izquierdo, M.S. (2007) Lipid accumulation in *Schizochytrium* G13/2S produced in continuous culture. *Appl Microbiol Biotechnol*, 76(5):985–990.

Gerster, H. (1998) Can adults adequately convert α-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int J Vitam Nutr Res*, 68:159-173.

Grynberg, A. (2003) Coeur et nutrition: Quels acides gras pour quelle fonction cardiaque? *Arch Mal Coeur Vaiss*, 96(6):7–12.

Hall, M.J. and Ratledge, C. (1977) Lipid-accumulation in an oleaginous yeast (*Candida* 107) growing on glucose under various conditions in a one-stage and two-stage continuous culture. *Appl Environ Microbiol*, 33:577–584.

Henderson, R.J., Mackinlay, E.E., Hodgsona, P.A. and Hardwood, J.L. (1990) Differential effects of the substituted pyridazinone herbicide Sandoz 9785 on lipid composition and biosynthesis in photosynthetic and non-photosynthetic marine microalgae. II. Fatty acid composition. *J Exp Bot*, 41:729–736.

Henderson, R.J. and Mackinlay, E.E. (1991) Polyunsaturated fatty-acid metabolism in the marine dinoflagellate *Crypthecodinium cohnii*. *Phytochemistry*, 30:1781–1787.

Jeffrey, B.G., Weisinger, H.S., Neuringer, M. and Mitchell, D.C. (2001) The role of docosahexaenoic acid in retinal function. *Lipids*, 36(9):859–871.

Klein, H.P. and Volkmann, C.M. (1975) Factors affecting palmitoylcoenzyme A desaturase of *Saccharomyces cerevisiae*. *J Bacteriol*, 124:718–723.

Lopes da Silva, T., Mendes, A., Mendes, R.L., Calado, V., Alves, S.S., Vasconcelos, J.M.T. and Reis, A. (2006) Effect of n-dodecane on *Crypthecodinium cohnii* fermentations and DHA production. *J Ind Microbiol Biot*, 3(6):408–16.

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

Meyer, F. and Bolch, K. (1963) Effect of temperatures on the enzymatic synthesis of unsaturated fatty acids in *Torulopsis utilis*. *Biochim Biophys Acta*, 77:671–673.

Moreton, R.S. (1988) Physiology of lipid accumulating yeasts. In: Moreton, R.S. (Eds.) Single cell oils, *The Bath Press*, Essex, UK, pp. 1–28.

Muskiet, F.A.J., Fokkema, M.R., Schaafsma, A., Boersma, E. R. and Crawford M. A. (2004) Is docosahexaenoic acid (DHA) essential? Lessons from DHA status regulation, our ancient diet, epidemiology and randomized controlled trials. *J Nutr*, 134:183–186.

Ratledge, C. (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, 86:807–815.

Rodríguez-Ruiz, J., Belarbi, E.H., Sánchez, J.L.G. and Alonso, D.L. (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotech Tech*, 12(9):689–691.

Roux, M.P., Kock, J.L.F., Du Preez, J.C. and Botha, A. (1995) The influence of dissolved oxygen tension on the production of cocoa butter equivalents and gamma-linolenic acid by *Mucor circinelloides*. *Syst Appl Microbiol*, 18:329–334.

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

Tsai, P.S., Rao, G. and Bailey, J.E. (1995) Improvement of *Escherichia coli* microaerobic oxygen metabolism by *Vitreoscilla* hemoglobin: new insights from NAD(P)H fluorescence and culture redox potencial. *Biotechnol Bioeng*, 47(3):347–354.

Valero, E., Milln, C. and Ortega, J.M. (2001) Influence of oxygen addition during growth phase on the biosynthesis of lipids in *Saccharomyces cerevisiae* (M330-9) in enological fermentations. *J Biosci Bioeng*, 92:33–38.

Van Pelt, C.K., Huang, M., Tschanz, C.L. and Brenna J.T. (1999) An octaene fatty acid, 4,7,10,13,16,19,22,25-octacosaoctaenoic acid (28:8n3), found in marine oils. *J Lipid Res*, 40:1501–1501.

Wang, Y., Crawford, M. A., Chen, J., Li, J., Ghebremeskel, K., Campbell, T. C., Fan, W., Parker, R. and Julius, L. (2003) Fish consumption, blood docosahexaenoic acid and chronic diseases in Chinese rural populations. *Comp Biochem Physiol A*, 136:127–140.

Yamane, Y., Higashida, K., Nakashimada, Y., Kakizono, T. and Nishio, N. (1997) Influence of oxygen and glucose on primary metabolism and astaxanthin production by *Phaffia rhodozyma* in batch and fed-batch cultures: kinetic and stoichiometric analysis. *Appl Environ Microb*, 63(11):4471–4478.

Yavin, E., Brand, A. and Green, P. (2002) Docosahexaenoic acid abundance in the brain: a biodevice to combat oxidative stress. *Nutr Neurosci*, 5:149–157.

Chapter 5

Restriction in propionic acid precursors inhibits odd-chain fatty acid synthesis in *Schizochytrium* G13/2S

This work, which remains un-published, was carried out at the fermentation laboratories of the University of Hull (England).

Abstract

The valuable polyunsaturated fatty acids present in *Schizochytrium* G13/2S increased from 47 to 52 % total fatty acids (TFA) in response to the concomitant decrease in the production of odd-chain fatty acids (OCFA). The production of OCFA was studied by providing different nitrogen sources in a glucose-mineral salts medium. *Schizochytrium* G13/2S produced both pentadecaenoic acid (15:0) and heptadecaenic acid (17:0) in quantities that depended on the nitrogen source employed. When grown in shake flask culture with a complex nitrogen source, consisting of a mixture of proteose peptone and yeast extract, the proportion of OCFAs was, depending on the initial concentration of the nitrogen sources, 6 and 11 % TFA. When propionyl-CoA precursors were ommitted in the medium by using glutamate or ammonia as the sole nitrogen source, OCFA were less than 2 % TFA, showing a concomitant increase in the proportion of other fatty acids, including the valuable docosahexaenoic acid (22:6n-3; DHA), which proportion decreased from 39 to 45 % TFA.

Introduction

Docosahexaenoic acid (DHA; 22:6 n-3) is a valuable polyunsaturated fatty acid (PUFA) usually found in marine organisms. This fatty acid plays an essential role in many aspects of human health and is necessary to attenuate the negative effects of fatty acid imbalance in Western countries diets (Muskiet *et al.*, 2004). Single cell oil obtained from the fermentation of *Schizochytrium* sp., a marine thraustochytrid, is being used as a nutraceutical ingredient to

enhance the DHA content in human diet (Barclay *et al.*, 2005). Under optimal culture conditions, this organism is capable of accumulating 50 % of its biomass as lipids with over 30 % DHA in total fatty acids (TFA) (Yokochi *et al.*, 1998).

This organism produces, along with DHA, variable amounts of pentadecaenoic (15:0) and heptadecaenoic (17:0) acids. Certain strains of *Schizochytrium*, grown in a glucose, yeast extract medium, could accumulate 15:0 up to about 39 % of TFA (Fan *et al.*, 2001), which subtracts from the valuable DHA accumulation. Moreover, the consumption of odd-chain fatty acids is contraindicated for patients with propionic acidemia (Feliz *et al.*, 2001). Odd-chain fatty acids (OCFA) are synthesised by the incorporation of propionate into the carbon chain, either as a primary building block or as an elongation unit (previous carboxylation to methylmalonyl). Cellular propionic acid is primarily generated from certain amino acids (valine, isoleucine, threonine, methionine) that are usually present in complex nitrogen sources (i.e. proteose peptone). Other culture conditions such as the carbon source employed (Tahoun *et al.*, 1988), temperature (Nichols and Russell, 1996), growth phase (Mannisto and Puhakka, 2001) or dilution rate (Jostensen and Landfald, 1996) could also affect OCFA content of the cell. In addition, medium supplementation with 0.05 g cyanocobalamin l^{-1} down-regulated odd-chain fatty acid formation in a variety of thraustochytrids including *Schizochytrium spp.* grown in yeast extract (Shirasaka *et al.*, 2005).

In order to reduce the OCFA and eventually enhance DHA content we studied the effect of different nitrogen sources on fatty acid profile of *Schizochytrium* G13/2S supplemented in a glucose-mineral medium.

Materials and Methods

Chemicals Monosodium glutamate was obtained by BDH (Poole, England); Thiamine, biotin, cyanocobalamin and nonadecanoic acid by Sigma; Proteose peptone and yeast extract from Oxoid.

Organism and culture conditions *Schizochytrium* sp. G13/2S, derived from G13 (Graham Bremer, University of Portsmouth, UK), was cultured in a completely defined medium (Ashford *et al.*, 2000) to which the nitrogen sources provided were modified. The medium was composed of ($g\ l^{-1}$): glucose (80), NaCl (12.5), $MgSO_4\ 7H_2O$ (2.5), KCl (0,5), $CaCl_2$ (0.1), KH_2PO_4 (0.5). This medium also contained trace-element solution ($5\ ml\ l^{-1}$) and vitamin solution ($1\ ml\ l^{-1}$). The trace element solution contained ($g\ l^{-1}$): EDTA di-sodium salt (6.0), $FeCl_3\ 6H_2O$ (0.29), H_2BO_3 (6.84), $MnCl_2\ 4H_2O$ (0.86), $ZnCl_2$ (0.06), $CoCl_2\ 6H_2O$ (0.026), $NiSO_4\ 6H_2O$ (0.052), $CuSO_4\ 5H_2O$

(0.002), Na_2MoO_4 $2H_2O$ (0.005). The vitamin solution contained ($mg\ l^{-1}$): thiamine (100), biotin (0.5) and cyanocobalamin (0.5). Nitrogen sources tested were ($g\ l^{-1}$): Glutamic acid mono-sodium salt (2.5), ammonium sulphate (1.65), ammonium tartrate (2), proteose peptone and yeast extract (as specified in each experiment).

Cultures were grown in shake flask (250 *ml*, 180 rpm) containing 50 *ml* medium at 27 °C. Medium was buffered with 1 M MOPS and pH was adjusted to 7 by addition of KOH. All culture components were autoclaved (15 *min*) prior to inoculation (10 % v/v). Three replicates by treatment were harvested 48 *h* after inoculation.

Determination of cell dry weight Samples of 20 *ml* where harvested by centrifugation (3,000 *g*) for 5 *min* at 10 °C. Biomass was washed twice by resuspension in demineralised water (10 °C) and centrifuged as before. The pellet fraction was freeze-dried and dry biomass was measured in a microbalance.

Fatty acid analysis Fatty acid methyl esters were obtained by the method described by Rodríguez-Ruiz *et al.* (1998) using nonadecanoic acid (19:0), previously dissolved in hexane, as internal standard. Fatty acid methyl esters were separated by GC with He as a carrier gas using a BPX70 25 *m* x 0.22 *mm* i.d. column (SGE). The temperature of the column was raised from 160 °C to 225 °C at 5 °C min^{-1} and then maintained at 225 °C for a further 5 *min*. Peak detection was performed by a flame ionisation detector. The temperature of the injection and detection ports was 250 °C. Fatty acids were identified by comparison to well-characterised external standards (Sigma).

Results

Schizochytrium G13/2S produced 15:0 and 17:0 when cultivated with the complex nitrogen source (see Figure 5.1A). The proportion of OCFA increased two-fold when 8 *g* proteose peptone l^{-1} and 5 *g* yeast extract l^{-1} were provided instead of 2 *g* proteose peptone l^{-1} and 1.25 *g* yeast extract l^{-1} in *Schizochytrium* G13/2S shake-flask cultures (Table 5.1). The low yeast extract and proteose peptone treatment provided a similar amount of nitrogen, but induced a higher production of OCFA than the treatments containing glutamate, ammonium tartrate or ammonium sulphate, indicating that propionate precursors were present in the complex nitrogen sources. Depending on the OCFA production, even-chain fatty acid percentage, polyunsaturated fatty acid and DHA proportion changed between 42–47, 47–55 and 39–45 % total fatty acids (TFA), respectively. OCFA were still synthesised (2 % TFA)

when providing a carbon-free nitrogen source such as ammonium sulphate, indicating that some propionate might be derived from cell anabolism.

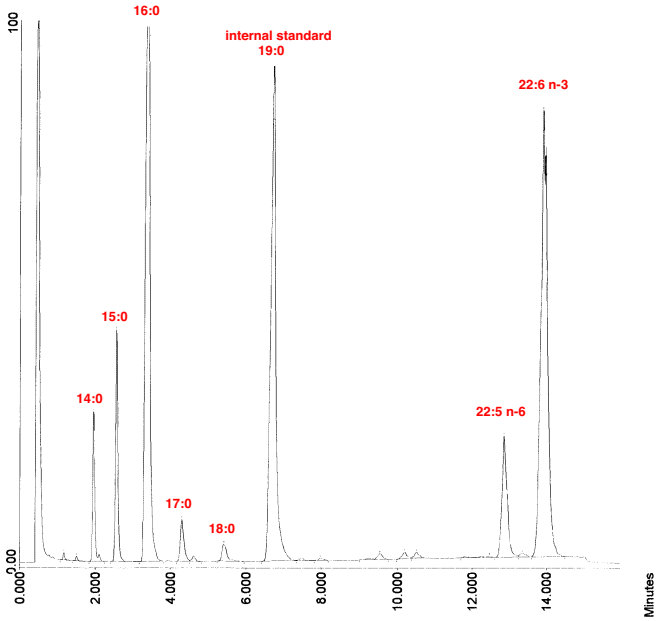
Table 5.1: Effect of nitrogen source in fatty acid classes of *Schizochytrium* G13/2S flask cultures.

FA classes	Nitrogen sources ($g\ l^{-1}$)				
(% TFA)	PP & YE	PP & YE	ammonium sulphate	ammonium tartrate	monosodium glutamate
	(8) & (5)	(2) & (1.2)	(2)	(1.65)	(2.5)
14:0	3	4	4	4	3
15:0	9	5	2	2	2
16:0	38	38	41	39	39
17:0	2	1	0	1	0
18:0	1	1	2	1	2
22:5 n–6	8	9	9	9	9
22:6 n–3	39	42	42	44	45
Polyunsaturated FA	47	51	51	53	54
Even-chain FA	42	43	47	45	44
Odd-chain FA	11	6	2	2	2

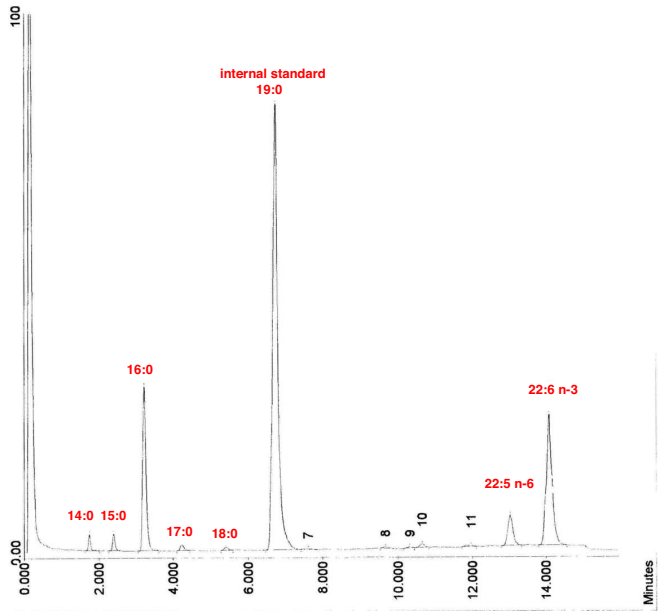
Medium contained 80 g glucose l^{-1}. Percentage values of the fatty acid classes in total fatty acids have been expressed on a weight-by-weight basis. Cells, harvested 48 h after inoculation, contained 20-30 % TFA in dry weight in all treatments. The figures have been quoted in order to include the error shown by three replicates (n=3). Abrv: PP=proteose peptone YE=yeast extract FA=Fatty acids.

Discussion

Prokariotic organisms are the main microbial sources of OCFA. Dairy products, due to the bacterial activity in the rumen of the cow, contain OCFA around 2 % TFA (Chouinard *et al.*, 1998; Vlaeminck *et al.*, 2006). Thraustochytrids, including *Schizochytrium* G13/2S, are also able to produce OCFA (15:0 and 17:0), certain strains are capable of accumulating up to 38 % TFA (Wang *et al.*, 2000; Fan *et al.*, 2001). OCFA accumulate in both neutral and polar lipid classes, particularly in phosphatidylcholine (Abe *et al.*, in press). The physiological implications of OCFA nutrition for humans are relatively unknown. However, the present experiment showed that the OCFA levels in *Schizochytrium* sp. can be decreased to those found in diary products. Therefore, the OCFA in this algae should not compromise their nutritional value. In addition, the decrease in OCFA production corresponds to increased DHA from 39 to 44 % TFA, which is the main production interest in this single cell oil. Thus, the present results confirm that 15:0 and 17:0



(a) Yeast extract & proteose peptone



(b) Ammonium tartrate

Figure 5.1: Gas chromatography (GC) chromatograms of fatty acid methyl esters obtained from *Schizochytrium* G13/2S grown in a glucose-medium containing different nitrogen sources. 14:0, tetradecanoic acid; 15:0, pentadecaenoic acid; 16:0, palmitic acid; 17:0, heptadecanoic acid; 18:0, stearic acid; 22:5 n-6, docosapentaenoic acid (DPA); 22:6 n-3 docosahexaenoic acid

accumulation can decrease the percentage of other fatty acids including the valuable DHA.

When peptone and yeast extract were present in *Thraustochytrium* sp. cultures, the cellular OCFA % TFA of increased. It has been suggested that their production was regulated by the yeast/peptone ratio (Wang *et al.*, 2000). Nevertheless, growing *Schizochytrium* sp. in a medium using yeast extract as the sole nitrogen source resulted in the accumulation of OCFA as high as 40 % (Fan *et al.*, 2001). The present study shows that the percentage of OCFA in *Schizochytrium* G13/2S lipids increases with the amount of complex nitrogen sources employed. The effect of yeast extract and proteose peptone concentrations in OCFA % of *Schizochytrium* sp. suggests that some propionate, or propionate precursors, that are actively being consumed as a carbon source, are present in these components. This C_3 molecule will be ultimately incorporated in the fatty acid synthesising machinery providing uneven carbon chains (Ingram *et al.*, 1977). Accordingly, we observe that through the implementation of defined nitrogen sources, containing no propionate precursors, the OCFA content decreased to the minimum amount of 2 % TFA that should be inherent to the specific metabolism of this organism. In summary, the medium developed in this study decrease 5-fold accessory OCFA in support of DHA, which increases from 39 to 44 %, which demonstrates the plasticity of the *Schizochytrium* G13/2S fatty acid profile. These results emphasise the relevance of developing a completely defined medium adjusted to the requirements the process.

Acknowledgements

This work was supported by the Tomás de Zárate Grant (Cabildo de Gran Canaria). We thank Colin Ratledge, Alistair Anderson and Simon Hopkins for their help and assistance in this work.

References

Abe, E., Hayashi, Y., Hama, Y., Hayashi, M., Inagaki, M. and Ito, M. (in press). A novel phosphatidylcholine which contains pentadecanoic acid and *sn*-1 and docosahexaenoic acid at *sn*-2 in *Schizochytrium* sp. F26-b. *J Biochem*, DOI 10.1093/jb/mvj145.

Ashford, A., Barclay, W.R. Weaver, C.A., Giddings, T.H. and Zeller, S. (2000) Electron microscopy may reveal structure of docosahexaenoic acid-rich oil within *Schizochytrium* sp. *Lipids*, 35:1377–1386.

Barclay, W., Weaver, C. and Metz, J. (2005) Development of a docosa-

hexaenoic acid production technology using *Schizochytrium*: a historical perspective. In: Ratledge, C. and Cohen, Z. (Eds.) Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 36–49.

Chouinard, P.Y., Girard, V. and Brisson, G.J. (1998) Fatty acid profile and physical properties of milk fat from cows fed calcium salts of fatty acids with varying unsaturation. *J Dairy Sci*, 81:471–481.

Fan, K.W., Chen, F., Jones, E.B.G. and Vrijmoed, L.L.P. (2001) Eicosapentaenoic and docosahexaenoic acids production by and okara-utilising potential of thraustochytrids. *J Indust Microbiol Biotech*, 27:199–202.

Feliz, B., Witt, D.R. and Harris, B.T. (2003) Propionic acidemia: a neuropathology case report and review of prior cases. *Arch Pathol Lab MEd*, 127(8):325–328.

Ingram, L.O., Chevalier, L.S., Gabba, E.J., Ley, K.D. and Winters, K. (1977) Propionate-induced synthesis of odd-chain-length fatty acids by *Escherichia coli*. *J Bacteriol*, 131(3):1023–1025.

Jostensen, J. P. and Landfald, B. (1996) Influence of growth conditions on fatty acid composition of a polyunsaturated-fatty-acid-producing *Vibrio* species. *Arch Microbiol*, 165:306–310.

Mannisto, M. K. and Puhakka, J. A. (2001) Temperature and growth-phase-regulated changes in lipid fatty acid structures of psychrotolerant groundwater Proteobacteria. *Arch Microbiol*, 177:41–46.

Muskiet, F.A.J., Fokkema, M.R., Schaafsma, A., Boersma, E.R. and Crawford M.A. (2004) Is docosahexaenoic acid (DHA) essential? Lessons from DHA status regulation, our ancient diet, epidemiology and randomized controlled trials. *J Nutr*, 134:183-186.

Nichols, D. S. and Russell, N. J. (1996) Fatty acid adaptation in an Antarctic bacterium - changes in primer utilisation. *Microbiology-Uk*, 142:747–754.

Rodríguez-Ruiz, J., Belarbi, E.H., Sánchez, J.L.G. and Alonso, D.L. (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotech Tech*, 12(9):689–691.

Shirasaka, N., Hirai, Y., Nakabayashi, H. and Yoshizumi H. (2005) Effect of cyanocobalamin and *p*-toluic acid on the fatty acid composition of *Schizochytrium limacinum* (Thraustochytriaceae, Labyrinthulomycota). *Mycoscience* 46:358–363.

Tahoun, M.K., Shata, O.H., Mashalley, R.I. and Abou-Domia, S.A. (1988) Influence of selected sugars and temperature on fatty acids composition in *Candida tropicalis*. *Nahrung*, 24(3):1432–0614.

Vlaeminck, B., Fievez, V., Tamminga, S., Dewhurst, R.J., van Vuuren, A., De Brabander, D. and Demeyer, D. (2006) Milk odd- and branched-chain fatty acids in relation to the rumen fermentation pattern. *J Dairy Sci*, 89:3954–3964.

Wang, Y., Kim, H. and Weete, J. D. (2000) Docosahexaenoic production by *Thraustochytrium aureum*. In *91st AOCS Annual Meeting & Expo*, San Diego, California.

Yokochi, T., Honda, D., Higashihara, T. and Nakahara, T. (1998) Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. *Appl Microbiol*, 49:72–76.

Chapter 6

High-cell-density cultivation of *Schizochytrium* G13/2S in an ammonium/pH-auxostat fed-batch system

This work was made at the University of Hull (England) and is being published in: Ganuza, E., Anderson, A.J. and Ratledge, C. (in press) *Biotechnol Lett.*

Abstract

Thraustochytrids, in particular *Schizochytrium spp.*, are targeted microorganisms for the production of the valuable docosahexaenoic acid (DHA). Growth of *Schizochytrium* sp. G13/2S in a defined medium was initially made in shake-flask cultures to determine the optimum concentrations of glucose (100–200 $g\,l^{-1}$) and ammonium (300 $mg\,l^{-1}$) that could be used by this microorganism. In subsequent fermenter culture, a pH-auxostat method was used to maintain NH_3 from 200–300 $mg\,l^{-1}$. During the first 49 h of fermentation, the 150 g glucose l^{-1} produced 63.3 g cell dry weight l^{-1}. Although growth was not limited by the supply of nitrogen, total fatty acids were at 25 % cell dry weight, which is more than half the lipid content of commercially-grown *Schizochytrium* biomass, which uses N-limited medium in the final stages for maximun ipid accumulation. This strategy is therefore useful for the cultivation of *Schizochytrium* to a high cell density up to the point when lipid accumulation can be triggered by N exhaustion from the medium.

Introduction

Polyunsaturated fatty acids (PUFA), particularly the very long chain ones of eicosapentaenoic acid (22:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3), are widely recognised as important nutritional components for the prevention of various cardiac disorders. The latter fatty acid, together with

arachidonic acid (ARA; 20:4 n-6), is also incorporated into infant formulae in many countries of the world because of their promotion of visual acuity and neural development (Sinclair *et al.*, 2005). Both DHA and ARA are produced exclusively for this purpose from microorganisms: *Crypthecodinium cohnii* and *Mortierella alpina*, respectively (Wynn and Ratledge *et al.*, 2007). DHA is also produce for the adult oil supplement market using a *Schizochytrium* sp. (Barclay *et al.*, 2005). The whole biomass of this organism, which is a thraustochytrid and was originally regarded as a marine fungus, may also be useful for the development of aquafeeds for farmed fish, which require a source of DHA for the development of fish fry (Izquierdo, 2005; Ganuza *el al.*, in press).

In order to achieve lipid accumulation in microorganisms, the culture medium is formulated so that the supply of nitrogen becomes exhausted part way through the culture, after which cell proliferation ceases but the carbon feedstock (usually glucose) continues to be assimilated. However, in large-scale cultivations, which can involve a series of fermenters of increasing volume up to 150–220 m^3, the cells are cultivated in a balanced medium so that the maximum biomass can be produced at each stage. It is therefore not until the cells are inoculated into the final fermenter, that *Schizochytrium* cultures will become N-limited, and consequently lipids will be accumulated (Ganuza and Izquierdo, 2007). Even here, it is not until the final stage of the batch run that this will occur. For reasons of commercial confidentiality, very little information is available about the details of the large-scale production of single cell oils.

Schizochytrium ssp. are marine thraustochytrids that produce about 35–40 % of total fatty acids (TFA) as DHA (Yokochi *et al.*, 1998). However, the use of this micro-heterotroph as a source of DHA is limited by its high production costs in comparison to the current fish oil prices. In order to reduce costs of microbial DHA production, higher volumetric productivities must be pursued by culture optimization and the use of inexpensive medium components. Growth rate, cell density and DHA content of the biomass are essential parameters contributing to an optimum DHA productivity (Sijtsma and de Swaaf, 2004). High-cell-densities are usually attained using fed-batch systems, as this technique avoids the high initial concentrations of a substrate that might be toxic or growth-inhibitory for the organism. Within fed-batch systems, the pH-auxostat was first applied to feed acidic or basic nutrients that can be supplied in response to medium pH derivation (Martin and Hempfling, 1976). Under a pH-auxostat system, the activity of the microorganism itself regulates the nutrient supply to fit the concentration that permits the fastest growth. This technique was successfully used for microbial DHA production using acetic acid as feeding nutrient for *Crypthe-*

codinium cohnii (Ratledge *et al.*, 2001; de Swaaf *et al.*, 2003). At the moment, *Schizochytrium* is successfully grown in batch cultures using glucose as carbon source and NH_4 as a nitrogen source (Barclay *et al.*, 2003; Yaguchi *et al.*, 1997). The aim of this work is to evaluate the capacity of this system to optimize growth under non-nitrogen source limiting conditions, as a first step for the commercial production of DHA-rich biomass.

Materials and methods

Organism and culture conditions *Schizochytrium* G13/2S derived from G13 (Mr Graham Bremer, University of Portsmouth, UK) was initialy cultured in flasks (250 ml) containing 50 ml complex medium [($g\ l^{-1}$): glucose (40), proteose peptone (8), yeast extract (5) sea salt (25) and MOPS (21)]. Further flask cultures were carried out in a defined medium containing ($g\ l^{-1}$): glucose (80), $(NH_4)_2C_4H_4O_6$ (1.65), NaCl (12.5), $MgSO_4\ 7H_2O$ (2.5), KH_2PO_4 (0.5), KCl (0.5), $CaCl_2$ (0.1) and MOPS (21) unless stated otherwise. This medium also contained trace-element solution (5 $ml\ l^{-1}$) and vitamin solution (1 $ml\ l^{-1}$), which were added after sterilization. The trace element solution contained ($g\ l^{-1}$): EDTA di-sodium salt (6.0),$FeCl_3\ 6H_2O$ (0.29), H_2BO_3 (6.84), $MnCl_2\ 4H_2O$ (0.86), $ZnCl_2$ (0.06), $CoCl_2\ 6H_2O$ (0.026), $NiSO_4\ 6H_2O$ (0.052), $CuSO_4\ 5H_2O$ (0.002), $Na_2MoO_4\ 2H_2O$ (0.005). The vitamin solution was filter-sterilised (1 μm) and contained ($mg\ l^{-1}$): thiamine (100), biotin (0.5) and cyanocobalamin (0.5). The medium used in the fermenter contained ($g\ l^{-1}$): glucose (150), $(NH_4)_2SO_4$ (1.65), NaCl (12.5), $MgSO_4\ 7H_2O$ (2.5), KH_2PO_4 (2.5), KCl (0,5), $CaCl_2$ (0.1) as well as trace element solution (10 $ml\ l^{-1}$) and vitamin solution (1 $ml\ l^{-1}$). Before autoclaving (121 °C, 15-30 min, glucose and $MgSO_4\ 7H_2O$ in a separate container) the pH of the medium was adjusted to 7 with KOH (2 M).

Shake flask cultures (180 rpm, grown for 24 h) containing either complex or defined medium were used as inoculum (10 % v/v) for shake-flask experiments and fermenter cultures, respectively. All cultures were grown at 27 °C and pH was maintained at 7 whenever possible. *Schizochytrium* G13/2S was finally cultivated in a 1 l working capacity bioreactor (Braun) in which the pH was controlled by the automatic addition of 10 % NH_4OH (10 % w/v). Dissolved O_2 was maintained over 30 % by manual increase of the stirring speed (Rushton blade impellers) from 300 to a maximum of 700 rpm. The aeration rate was always 0.5 vol. air (vol. medium)$^{-1}$ min^{-1}, previously filtered through a 0.2 μm sieve. Addition of antifoam was not necessary during fermentation.

Determination of cell dry weight (CDW) Culture samples (20 ml) where harvested by centrifugation (3000 g, 5 min, 10 °C) and biomass was washed twice in demineralised water. The pellet fraction was freeze-dried and dry biomass was measured in a microbalance.

Lipid analysis Fatty acid methyl esters were prepared according to the method described by Rodríguez-Ruiz *et al.* (1998) with nonadecanoic acid as internal standard. FAMEs were separated by GC with He as a carrier gas using BPX70 25 m x 0.22 mm i.d. column (SGE). The temperature of the column was raised from 160 °C to 225 °C at 5 °C min^{-1} and then maintained at 225 °C for further 5 min. The temperature of the injection port and flame-ionisation port was 250 °C. Fatty acids were identified by comparison to well characterised external standards (Sigma).

Glucose and ammonia determination The residual glucose concentrations were analysed using a glucose oxydase method (Boehringer Mannheim) and ammonia by the indophenol-based method.

Results

Optimum ammonium concentration *Schizochytrium* G13/2S was grown in shake-flask cultures on a defined medium with glucose and diammonium tartrate as principal carbon and nitrogen sources. It could grow without impediment with ammonium tartrate up to 13.2 $g\ l^{-1}$ (2438 mg $NH_4\ l^{-1}$) even though the final pH of the medium was below 4 (see Table 6.1). The drop in pH was not controllable even when the medium was strongly buffered with 0.25 M MOPS. [Diammonium tartrate was used in preference to $(NH_4)_2SO_4$ in order to prevent an even greater fall in pH.] The low pH, however, did not appear to be detrimental to growth.

Optimum glucose concentration The maximum glucose concentration that sustained optimum growth was between 100 and 200 $g\ l^{-1}$ (see Figure 6.1). In these experiments, sodium glutamate was used as N source as this prevented a decrease in pH which then remained at 6.8–6.9 during growth. (It would have, however, been inappropriate to use this as a nitrogen source in the previous experiments as it clearly is not a direct source of ammonium ions.)

NH_4/pH-auxostat fermentation Having determined the concentrations of glucose and ammonia (150 $g\ l^{-1}$ and 2.4 $g\ l^{-1}$) to which *Schizochytrium*

Table 6.1: Influence of the initial ammonium tartrate concentration (0–13.2 $g\ l^{-1}$) on cell dry weight (CDW) and pH (in parenthesis) of *Schizochytrium* G13/2S in shake-flask cultures.

Time (h)		Ammonium tartrate ($g\ l^{-1}$)				
		0	1.7	3.3	6.6	13.2
0[b]	CDW	0.4	0.4	0.4	0.4	0.4
	pH	(6.8)	(6.8)	(6.8)	(6.8)	(6.8)
24	CDW	1.6	6.6	7.7	8.9	8.4
	pH	(6.4)	(6.6)	(4.4)	(4.1)	(4.1)
48	CDW	1.8	10.4	18.5	22.0	23.8
	pH	(6.6)	(5.9)	(4.4)	(3.5)	(3.2)

[a] The basal medium was strongly buffered with 0.25 M MOPS. Represented data are the average value of two independent replicates. [b]Inoculation time (0 h).

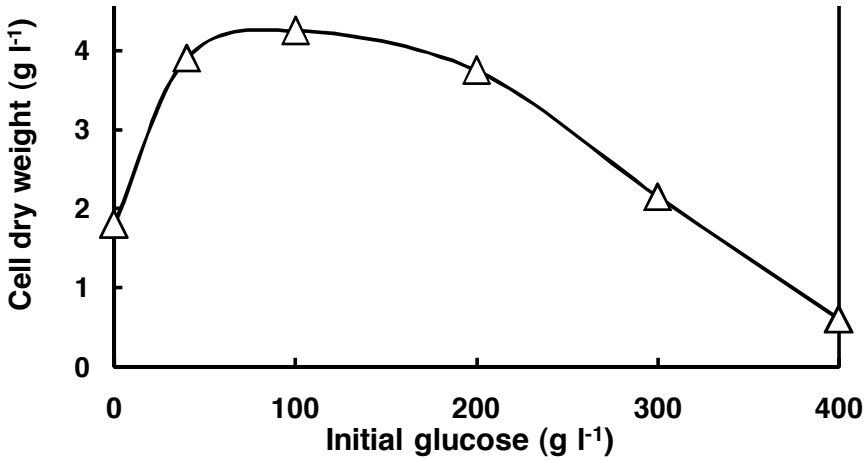


Figure 6.1: Influence of initial glucose concentration in 24 h growth of *Schizochytrium* G13/2S in shake-flask-culture. *Represented data are the average value of two independent replicates maintained at a constant pH of 6.9 through the implementation of glutamic acid sodium salt (3 $g\ l^{-1}$) as sole nitrogen source

sp. was tolerant, a pH-auxostat fermentation was initiated in which NH_4OH was added to control the pH at 7. During a typical fermentation run (see Figure 6.2) some 135 ml 9.5 % (w/v) NH_4OH (= 6.2 g NH_3) were added over 49 h providing a biomass yield of 10.2 g CDW g^{-1} NH_3. The ammonia concentration within the fermenter was maintained between 300 and 400 mg

l^{-1} by this strategy (Figure 6.2B). Thus, *Schizochytrium* G13/2S could be grown under non-nitrogen limiting conditions to a high biomass density.

Glucose, initially at 150 $g\ l^{-1}$, was readily utilized during the fermentation (see Figure 6.2A) and by 48-49 h had been totally consumed producing 63.3 g CDW l^{-1} (= 0.42 g CDW g glucose^{-1}). As no further glucose was added into this fermentation medium, growth then ceased with glucose depletion. However, in other fermentation runs, further glucose was added after 60 h to give 65 $g\ l^{-1}$, but this only increased the cell density from 60 $g\ l^{-1}$ to 65 $g\ l^{-1}$ after a further 50 h cultivation (data not shown).

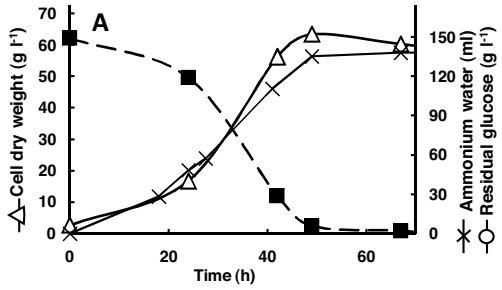
As the cultures were grown without any nutrient limitation, including nitrogen, the cells did not accumulate much lipid during the course of the fermentation run (see Figure 6.2B). This was in accordance with expectation as lipid accumulation is triggered by the exhaustion of a nutrient other than carbon (usually this is nitrogen) (see Ratledge and Wynn (2002)). The total fatty acid content of the cells remained at about 25 % throughout growth and thus was entirely growth-associated (see Figure 6.2C). The profile of fatty acids (see Table 6.2) remained approximately constant throughout growth though there were small changes in the levels of palmitic acid (16:0) and DHA (22:6 n-3). At the end of growth (49 h), the profile of the fatty acids was similar to that reported for the SCO derived from a commercial strain of *Schizochytrium* (Barclay *et al.*, 2005) in which the long chain fatty acids (DHA and docosapentaenoic acid, 20:5 n-6) were, respectively, 17 and 41 %.

Thus, polyunsaturated fatty acid synthesis is occurring in cells undergoing balanced growth and is not specifically engendered by nitrogen becoming limited in the medium. This accord with previous suggestions that lipid accumulation does not occur by increased activity of lipid-synthesising enzymes after nitrogen limitation is reached but by other enzyme activities associated with cell growth and proliferation ceasing (Ratledge and Wynn, 2002).

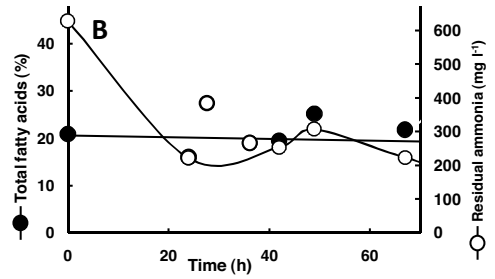
The overall performance of the cells grown without nitrogen limitation is shown in Figure 6.2; there was no limitation of O_2 to the cultures (see Figure 6.2D) and microscopic examination of the cells made at the highest agitation rate (700 rpm) did not reveal any damage due to shear stress. The overall DHA volumetric productivity (r_DHA) was 125 mg DHA $l^{-1}h^{-1}$. This value is over twice the values recorded for the production of DHA using *Crypthecodinium cohnii*, the other commercial DHA-producing microorganism (see Sijtsma *et al.* (2005)).

Discussion

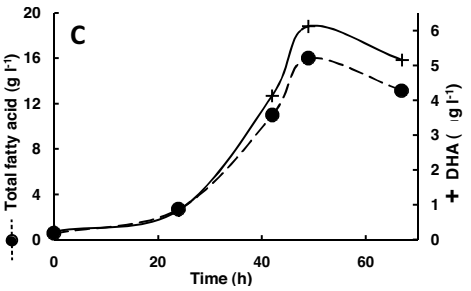
Schizochytrium spp. are commercially cultivated in batch culture with a nitrogen-limiting medium. Under these conditions, cell yields above 200 g



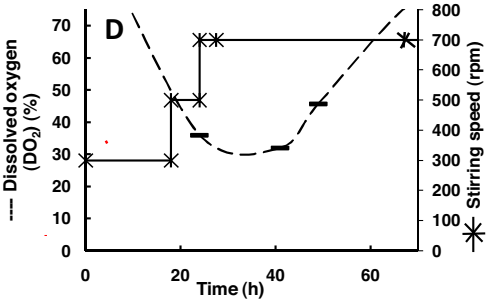
(a) Biomass production (■), residual glucose (△) and ammonium water addition (×).



(b) Residual ammonia (○) and total fatty acids accumulation (●).



(c) DHA (+) and total fatty acid production (-●-).



(d) Dissolved oxygen (-\-) and stirring speed (✳).

Figure 6.2: The pH-auxostat cultivation of *Schizochytrium* G13/2S fed with 5 % w/w ammonium water. The initial medium contained 150 g glucose l^{-1} and 10 % v/v inoculum. Represented data are the typical values of multiple fermentation carried under the similar conditions.

CDW l^{-1} containing around 40 % lipids have been claimed (Barclay *et al.*, 2003) although this is not likely to be attained in the commercial process because of difficulties in pumping out the cells from the fermenter and subsequent down-stream processing. This present study was carried out using non-limiting nitrogen conditions as the ammonium consumed by the organism was automatically replaced by the pH-control. Although this condition does not induce lipid accumulation (which needs exhaustion of the N source in order to channel the carbon flux from glucose into fatty acids rather than other cell components), any large-scale production process for a Single Cell Oil will use a sequence of ever-larger fermenters in which the objective is to grow the cells as quickly as possible and to the highest practical density. It will not be until the final stage is reached that the medium will be formulated to become N-limiting. Up to this point, the medium will be designed

Table 6.2: Fatty acid profile (% of TFA) of *Schizochytrium* G13/2S during in a NH_4/pH-auxostat culture.

Fatty acids residues[a]	Time (h)				Commercial oil[b]
	0	24	42	49	
14:0	3	4	4	4	7
16:0	29	54	43	43	22
18:0	1	2	3	3	1
22:5 n–6	13	6	8	8	17
22:6 n–3	44	31	38	38	41
Minor fatty acids[c]	10	3	4	4	13

[a] Fatty acid residues values are represented in relative weight % of total fatty acids. The experiment was repeated once and each sample was analyzed in triplicate.
[b] Obtained from Barclay *et al.* (2005)
[b] In all samples small amounts (in total < 2 %) of 17:0, 15:0 were detected.

to ensure maximum biomass (not lipid) production i.e. will use a balanced composition of nutrients. And this is therefore what we have done here.

Flask and fermenter cultures demonstrated that both glucose and ammonium were well utilised by *Schizochytrium* strain G13/2S. In agreement with the work of Yaguchi *et al.* (1997), we demonstrated that ammonium (as its tartrate salt) was not toxic at 30 gl^{-1}, however, the growth rate was faster with low concentrations of NH_4^+ (aprox. 1.7 gl^{-1}), indicating that an NH_4^+ fed-batch cultivation would be advantageous for attaining the fastest growth rate. Accordingly, the biomass productivity attained in this experiment (1.3 g CDW $l^{-1}h^{-1}$) was at least twice the productivity obtained by Yaguchi and co-workers (0.53 g CDW $l^{-1}h^{-1}$). Although the organism tolerates high concentrations of glucose (around 150 gl^{-1}), these may not be necessarily sufficient to attain the optimal cell densities (60–80 gl^{-1}) and maximal lipid contents (40–45 %) during commercial production, suggesting that a fed-batch culture of glucose would also be beneficial. Although the lipid accumulation was not pursued in this experiment, because of the very fast growth rates that were attained, DHA productivities were nevertheless still within the range described by Yaguchi *et al.* (1997) using a nitrogen-limited medium to optimize lipid, and therefore DHA, accumulation. The total fatty acid content in these experiments was 25 % CDW, indicating that more than half the lipid content of the commercially available *Schizochytrium*

sp. biomass had already been reached in the first phase of the culture Barclay *et al.* (2005). Thus, once the cell density in the fermenter had reached, say, 50 $g\ l^{-1}$ with 25 % lipid, a simple switching of alkaline for pH balance from NH_4OH to KOH would result in the cells entering the lipid accumulation phase in which the lipid would increase to its final value of about 45 %. This would result in a final biomass yield increase mainly due to increased lipid content of the cells.

We would also point out that we have been able to grow this species of *Schizochytrium* on an entirely chemically-defined medium using only NaCl to maintain an adequate osmotic pressure and not using artificial seawater salts that are so often used with these marine organisms. Avoidance of complex medium constituents, such as peptones or yeast extracts, also avoids introducing proteins or amino acids from which propionic acid may be derived (see 5). If this should be produced, it is readily incorporated into lipids to give odd-chain length fatty acids, which can occur in this organism to some small extent (Shirasaka *et al.*, 2005).

In conclusion, the NH_4/pH-auxostat system is proposed as a promising technique for the first stage production of *Schizochytrium* sp. due to the capacity to sustain fast growth and simultaneously produce a considerable amount of lipids and DHA.

Acknowledgements

This work was partialy supported by the Tomás de Zárate Grant (Cabildo de Gran Canaria). We thank Marisol Izquierdo for his help and assistance in this work.

References

Barclay, W.R., Bailey, R.B., DiMasi, D., Hansen, J.M., Mirrasoul, P.J., Ruecker, C.M., Veeder III, G.T. and Kaneko, T. (2003) Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermentors. United States Patent: 09,771,352, *Martek Biosciences Corporation*, Boulder, Columbia, pp. 1–12.

Barclay, W., Weaver, C. and Metz, J. (2005) Development of a docosahexaenoic acid production technology using *Schizochytrium*: a historical perspective. In: Ratledge, C. and Cohen, Z. (Eds.) Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 36–49.

de Swaaf, M.E., Sijtsma, L. and Pronk, J.T. (2003) High-cell-density

fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohnii*. *Biotech Bioeng*, 81(6):666–672.

Ganuza, E., Benítez-Santana, T., Atalah, E., Vega-Orellana, O., Ganga, R., Hernández-Cruz, C.M. and Izquierdo, M.S.(in press) *Crypthecodinium cohnii* and *Schizochytrium* sp. as potential substitutes to fisheries derived oils in *Sparus aurata* microdiets. *Aquaculture*.

Ganuza, E. and Izquierdo, M.S. (2007) Lipid accumulation in *Schizochytrium* G13/2S produced in continuous culture. *Appl Microbiol Biotechnol*, 76(5):985–990.

Izquierdo, M.S. (1996) Essential fatty acid requirements in Mediterranean fish species. *Cah Options Mediterr*, 63:91–102.

Martin, A.A. and Hempfling, W.P. (1976) A method for the regulation of microbial population density during continuous culture at high growth rates. *Archiv Microbiol*, 107:41–47.

Ratledge, C., Kanagachandran, K., Anderson, A.J., Grantham, D.J. and Stephenson, J. C. (2001) Production of docosahexaenoic acid by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. *Lipids*, 36(11):1241–1246.

Ratledge, C., Wynn, J.P. (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbiol*, 51:1–51.

Rodríguez-Ruiz, J., Belarbi, E.H., Sánchez, J.L.G. and Alonso, D.L. (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotech Tech*, 12(9):689–691.

Sijtsma, L., Anderson, A.J. and Ratledge, C. (2005) Alternative carbon sources for heterotrophic production of docosahexaenoic acid by the marine algae *Crypthecodinium cohnii*. In: Ratledge, C. and Cohen, Z. (Eds.) Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 107–130.

Sijtsma, L. and de Swaaf, M.E. (2004) Biotechnological production and applications of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. *Appl Microbiol Biotech*, 64(2):146–153.

Sinclair, A., Attar-Bashi, N., Jayasooriya, A., Gibson, R., Makrides, M. (2005) Nutritional aspects of single cell oils: uses and applications of arachidonic acid and docosahexaenoic acid oils. In: Ratledge, C. and

Cohen, Z. (Eds.) Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 182–201.

Shirasaka, N., Hirai, Y., Nakabayashi, H., and Tyshizumi, H. (2005) Effect of cyanocobalamin and *p*-toluic acid on the fatty acid composition of *Schizochytrium limacinum* (Thraustochytriaceae, Labyrinthulomycota). *Mycoscience*, 46:358–363.

Wynn, J.P., Ratledge, C. (2007) Microbial oils: production, processing and markets for specialty long-chain omega-3 polyunsaturated fatty acids. In: Breivik, H. (Ed) Long-chain omega-3 spacialty oils, *The Oily Press*, Bridgwater, England, 1^{st} edition, pp. 43–72.

Yaguchi, T., Tanaka, S., Yokochi, T., Nakahara, T. and Higashihara, T. (1997) Production of high yields of docosahexaenoic acid by *Schizochytrium* sp. strain SR21. *J Am Oil Chem Soc*, 74:1431–1434.

Yokochi, T., Honda, D., Higashihara, T. and Nakahara, T. (1998) Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. *Appl Microbiol Biotech*, 29:72–76.

Chapter 7

Docosahexaenoic acid production by *Crypthecodinium cohnii* grown in an ethanol fed-batch system

This work, which remains un-published, was carried out at the fermentation laboratories of the University of Hull (England).

Abstract

The heterotrophic dinoflagellate *Crypthecodinium cohnii* synthesises the valuable nutritional docosahexaenoic acid (DHA; 22:6 n-3) with polyunsaturated fatty acid present in less than 1 %. Its potential to produce DHA in fermenter was studied using an ethanol fed-batch system according to the method of Swaaf *et al.* [(2003a) Applied Microbiology and Biotechnology 61: 40-43]. The strain ATCC 50060 was cultured in a 10 l working capacity fermenter producing 91 g cell dry weight l^{-1}, 31 g total fatty acid l^{-1} and 12 g DHA l^{-1} after 210 h of fermentation. The overall DHA volumetric productivity the strain was 56 mg DHA l^{-1} h^{-1} (using 10 % inoculum), slightly higher than that previously reported (53 mg DHA l^{-1} h^{-1}) for this microalga. Since the dissolved O_2 between 93 and 160 h of fermentation was under 5 % of saturation, further optimization of the culture was attempted through the automatic substitution of the air with pure O_2. By this means, the saturation was maintained above 30 % all along the fermentation, but the growth, fatty acid accumulation and DHA concentration obtained were not further enhanced, indicating that dissolved O_2 was not a growth-limiting factor. Ethanol-fed-batch cultivation is confirmed to be the most productive cultivation system so far and further optimization of the method in the 10 l fermenter should be focused on parameters different from the O_2, such as the medium composition and the strain (main improvement of the present experiment).

Introduction

Docosahexaenoic acid (DHA, 22:6 n-3) is an important highly unsaturated fatty acid in human and fish nutrition. It is involved in the prevention

of several diseases such as cardiovascular diseases (Lichtenstein, 2003) and hormone-dependent cancers (Terry *et al.*, 2003). It is also considered essential for fish larvae survival (Izquierdo *et al.*, 2000), fish reproduction (Izquierdo *et al.*, 2001) and fish health (Montero *et al.*, 2004). The main dietary source of this nutrient is the seafood for humans and the fish oil for aquacultured species. Nevertheless, the dwindling fish stocks are not expected to meet the fish oil demand from the growing aquaculture sector (Sargent and Tacon, 1999). In order to maintain the delivery of this fatty acid in human diet also with the aquaculture products, alternative sources of DHA to fish oil should be implemented as aquafeed ingredient. Unconventional microbial oils are investigated in order to produce clean, sustainable and reliable DHA-rich biomass (Ratledge, 2002) that eventually enables a sustainable growth of the aquaculture sector.

Crypthecodinium cohnii is marine dinoflagellate that, under adequate culture conditions, can accumulate about 50 % of its dry biomass as lipids containing up to 32 % DHA as the only highly unsaturated fatty acid (de Swaaf *et al.*, 2003b). The capacity to produce highly concentrated DHA-biomass is the main advantage of microbial sources over the bulk fish oil source of DHA actually used in aquaculture. Due to the nutritional requirements for concentrated DHA sources by marine fish, shrimp and mollusc larvae, microbial sources are gaining importance over fish oils in marine hatcheries. Thus, the fermentation feasibility mainly depends upon the DHA volumetric productivity (r_{DHA}) of the fermentation process (Sijtsma *et al.*, 1998). This parameter is determined by the cell density, the lipid content of the biomass, the DHA content of the lipids and the fermentation time. The lipid and DHA content of the biomass can be influenced by different parameters including the organisms, the dissolved O_2 (Lopes da Silva *et al.*, 2006; Roux *et al.*, 1995) and the carbon source (Sijtsma *et al.*, 2005). So far, acetic acid and ethanol based fed-batch cultures are the most successful fermentations. Acetic acid/pH-auxostat produced 36 mg DHA l^{-1} h^{-1} from a 5 % (v/v) of inoculum in 98 h of culture (Ratledge *et al.*, 2001) and 44.5 mg DHA l^{-1} h^{-1} in 210 h of culture (de Swaaf *et al.*, 2003b). Nevertheless, the highest productivity reported for *C. cohnii*, 53 mg l^{-1} h^{-1} using 10 % (v/v) inoculum, was attained with an ethanol fed-batch system (de Swaaf *et al.*, 2003a). The aim of the present experiment was to implement this method and optimised it using a new strain, *C. cohnii* ATCC 50060, and increasing the dissolved O_2 along fermentation.

Materials and methods

Chemicals Proteose peptone and yeast extract were obtained from Oxoid. Sea salts and heptadecanoic acid were supplied by Sigma. Polypropylene glycol-2000 grade was obtained from Fisher Scientific (Loughboough, England).

Organism and culture conditions. *Crypthecodinium cohnii* ATCC 50060 was incubated (3-10 d) at 27 °C in static flasks (250 ml) filled with 100 ml of medium. The medium of the standing cultures contained ($g\ l^{-1}$): sea salts (25), glucose (9) and yeast extract (2) at pH 6.5. Sub-cultures were used to inoculate shake-flask cultures (120 rpm, 27 °C) filled with 50 ml. The medium used in shake-flask cultures contained ($g\ l^{-1}$): sea salts (25), glucose (27) and yeast extract (3.8) at pH 6.5. Shake flask cultures incubated during 4 d were used to inoculate the fermenter. The inoculum size used was always 10 % v/v. All medium components were autoclaved maintaining glucose separately.

Fermentation was carried in a 10 l bioreactor (Bioflo 3000, New Brunswick). The initial medium contained ($g\ l^{-1}$): yeast extract (10), sea salts (25) and ethanol (5.5). During fermentation, 10 ml of polypropylene glycol-2000 antifoam agent was added to the culture. The pH was maintained at 6.5 ±0.5 by automatic addition of NaOH (2 M) or H_2SO_4 (2 M) while the temperature was set to 27 ±0.5 °C.

Stirring speed was programmed to automatically increase (but not decrease) from 200 up to a maximum of 1000 rpm in response to dissolved O_2 levels below 30 %. The fermenter contained two six-blade turbine impellers and four straight baffles, each of them having a width of 1/10 of the vessel diameter. Once the maximum stirring speed was attained, either the initial airflow was manually increased from 5 to 10 $l\ min^{-1}$ or the airflow was programmed to maintain dissolved O_2 levels above 30 % through the automatic substitution of the air by high purity O_2 (Engineering and Welding, Hull, UK).

Dissolved O_2 output (or alternatively the degree of air substitution) was used to evaluate the ethanol demand by the culture. Initially, when the residual ethanol was consumed (dissolved O_2 increasing above 35 % saturation), an initial feeding rate of 0.9 ml ethanol $l^{-1}\ h^{-1}$ was set with a flow inducer. The feeding rate was increased every time that an extra pulse of ethanol was automatically consumed by the culture (dissolved O_2 decreasing below 30 %). The maximum feeding rate attained was maintained until the end of the fermentation. Whenever the dissolved O_2 rose above 35 %, the ethanol feeding was stopped temporally in order to avoid growth inhibition.

Determination of cell dry weight (CDW). Culture samples (20-40 ml) where harvested by centrifugation (3000 g, 5 min, 10 °C) and biomass was resuspended in demineralised water. The pellet fraction of the second centrifugation was freeze-dried and dry biomass contained in a pre-weighted flask was measured in a microbalance. Calculations of the biomass yields were made after correction for sampling.

Lipid analysis. Fatty acid analysis of *C. cohnii* freeze-dried biomass (25-30 mg) was made using heptadecanoic acid (2.5 mg per sample), previously dissolved in methanol:chloroform (3:1), as internal standard,. Fatty acid methyl esters were prepared according to the method described by Rodríguez-Ruiz *et al.* (1998). Fatty acid methyl esters were separated by GC with He as a carrier gas using BPX70 25 m x 0.22 mm i.d. column (SGE). The temperature of the column was raised from 160 °C to 225 °C at 5 °C min^{-1} and then maintained at 225 °C for further 5 min. Peak detection was performed by a flame ionisation detector. The temperatures of the injection and detection ports were 250 °C. Fatty acids were identified by comparison to well characterised external standards (Sigma).

Results

Optimum ammonium concentration. *C. cohnii* ATCC 50060 is able to grow in an ethanol fed-batch culture with an initial concentration of 5 g ethanol l^{-1}. Dissolved O_2 was found to respond instantly to ethanol excess or limitation. After 25 h of culture, initial ethanol was consumed and a feeding rate of 0.9 ml ethanol l^{-1} h^{-1} was applied. From then onwards, ethanol-feeding rate was increased progressively until a maximum feeding rate of 2 ml ethanol l^{-1} h^{-1} that was maintained from 92 h of cultivation until the end of fermentation. All over the fermentation (210 h), 3 l of ethanol was added (Figure 7.1A). The biomass yield on ethanol, after correcting the loss of biomass caused by sampling, was 0.31 g biomass (g ethanol)$^{-1}$.

The lipid content of the cells increased constantly from 41 until 210 h of fermentation to a final value of 34 % TFA (Figure 7.1B). The DHA content of the lipid varied between 35–46 % and was finally 38 %. The fatty acid profile remained constant after 41 h of culture. The concentrations of biomass, TFA and DHA were 91 g CDW l^{-1}, 31 g TFA l^{-1} and 12 g DHA l^{-1} at 210 h (Figure 7.1C). For the inoculum size used (10 % v/v), the r_{DHA} in air-based fermentation was 56 mg DHA l^{-1} h^{-1} after 210 h of culture.

Between 92 and 170 h of cultivation, air-based fermentation contained dissolved O_2 level below 5 %. Other fermentations were performed under the same conditions but maintaining dissolved O_2 level over 30 % of saturation all

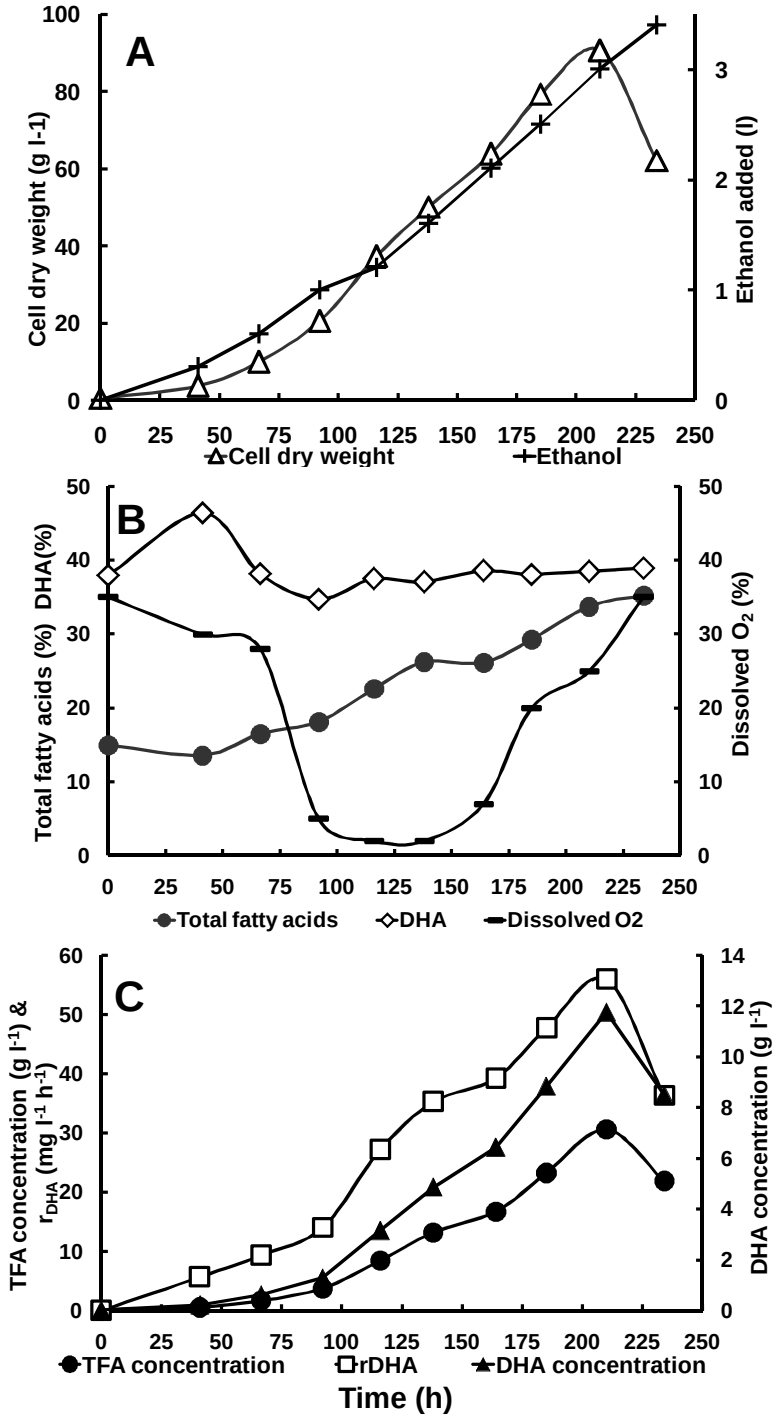


Figure 7.1: Fed-batch cultivation of *Crypthecodinium cohnii* ATCC 50060 in ethanol with a 10 *l* working capacity bioreactor.

along the culture by partial substitution of the air supply with pure O_2. The fermentation time in oxygenated system decreased to approximately 192 h. The performance in terms of biomass (Figure 7.2A), lipid production (Figure 7.2B) and DHA synthesis was equivalent in both aerated and oxygenated systems, showing no improvement due to O_2 supply. The lipid profile was also equivalent with myristic acid (14:0), palmitic acid (16:0), oleic acid (18:1) and DHA accounting for more than 90 % of total fatty acids (Table 7.1). Biomass yield in ethanol was slightly smaller (0.27 $\pm$0.01 g ethanol^{-1}) comparing to the first fermentation in which culture was only aerated (0.32 g ethanol^{-1} at 185 h).

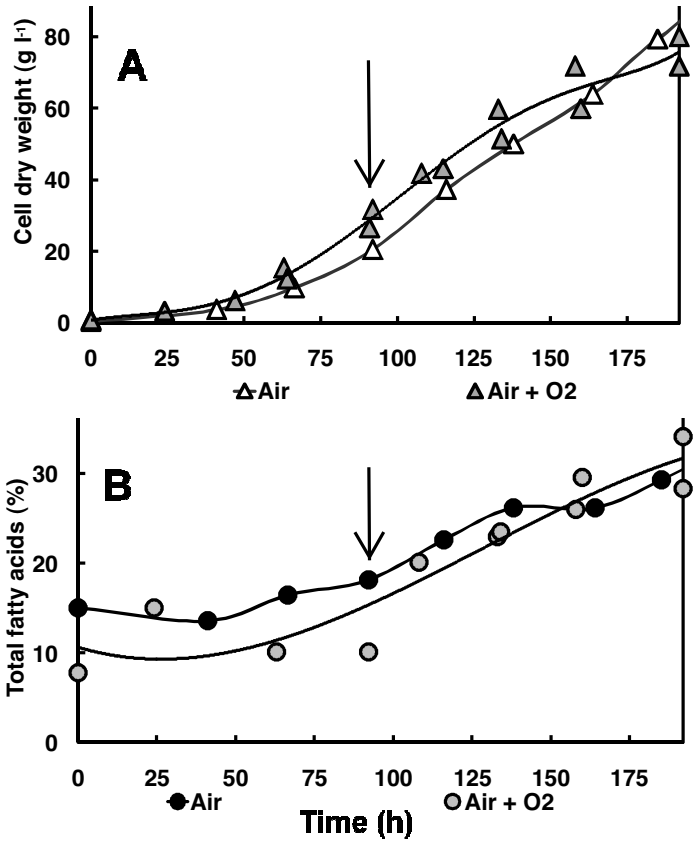


Figure 7.2: Growth and lipid accumulation in an ethanol fed-batch cultivation of *Crypthecodinium cohnii* under normal aeration and O_2-enriched aeration (two replicates). Between 92 ($\downarrow$) and 160 h of fermentation, the dissolved O_2 level under non-enriched aerating conditions was below 5 % of saturation.

Table 7.1: Fatty acid profile of *Crypthecodinium cohnii* growth in ethanol fed-batch culture under air and O_2-enriched air supply.

Fatty acids residues[a]	Air	Air + O_2	Commercial oil[b]
Time (h)	210	192	(Proprietary)
12:0	2	1	0–6
14:0	16	18	5–20
16:0	26	27	5–20
18:1	16	15	10–40
22:6 n–3	39	38	40–45
Other FA	1	1	-

[a] Represented values are typical from multiple fermentations carried out under similar conditions. Fatty acid residues values are given in relative weight % of total fatty acids.

[b] Wynn *et al.* (2005)

Discussion

Ethanol is a promising carbon source for the production of DHA by *C. cohnii*. The biomass yields on ethanol are 2.4 times higher than the yield on acetic acid (de Swaaf *et al.*, 2003b). Besides, it is relatively cheap and less corrosive than acetic acid. As two-carbon molecule it is a direct precursors of acetyl CoA, the basic building block for the fatty acid synthase (FAS) complex and consequently it is considered an ideal substrate for lipid synthesis (Sijtsma *et al.*, 2005). In this experiment, however, the fatty acid content was slightly lower than the expected for C_2 substrates (de Swaaf *et al.*, 2003b) indicating that the good productivities of this ethanol-based fermentation were sustained by the fast growth of the present strain, rather than by the increase in lipid or DHA accumulation in the cell.

C. cohnii is believed to have a very high O_2 demand, which contribute, under O_2-limitation, to decrease its lipid content (see Chapter 4). Dissolved O_2 could be a limiting factor in high-cell-density cultures and the fermentation scaling-up process will more likely increase this problem, since bigger vessels usually have lower O_2 transfer coefficient (KLa) (Cheng, 1998). In the present experiment we used a 10 l bioreactor in an attempt to produce enough biomass for aquaculture trialing. During most of the fermentation, dissolved O_2 was below 5 %, suggesting that O_2 was probably limiting growth, lipid and DHA synthesis of the system. Nevertheless, when fermentation was re-

Table 7.2: Comparison of *Crypthecodinium cohnii* acetic acid and two ethanol fed-batch cultures.

	Feeding substrate		
Strain (*C. cohnii*)	Acetic acid ATCC 30772[a]	Ethanol ATCC 30772[a]	Ethanol ATCC50060
Fermentation time (h)	210	200	210
Fermenter capacity (l)	2	2	10
Cell dry weight (CDW) ($g\ l^{-1}$)	59 ±3	77 ±3	91
Total fatty acid (TFA) (%)	50 ±1	41 ±1	34
DHA (% TFA)	32 ±0	33 ±0	39
TFA concentration ($g\ l^{-1}$)	30 ±1	31 ±1	34
DHA concentration ($g\ l^{-1}$)	9.5 ±0.1	10.1 ±0.4	12
Biomass yield (g (g substrate)$^{-1}$)	0.13	0.31	0.31
DHA productivity (r_{DHA})	44.5 ±0.5	51.4 ±2.1	57
Maximum feeding rate ($ml\ l^{-1}\ h^{-1}$)	pH-auxostat	3	2

[a] Data from ATCC 30772 obtained from de Swaaf *et al.* (2003a,b) is the average of two fermentations. Data were calculated for the data point representing the end of the fermentation.

peated maintaining dissolved O_2 supply above 30 % of saturation, no further improvement of growth or DHA accumulation were observed. Ethanol was consumed at the same maximum feeding rate (2 ml ethanol $l^{-1}\ h^{-1}$) as the non O_2 enriched fermentation. These results suggest that some other limiting factor may be acting in the culture.

Crucial parameters for the fed-batch in ethanol obtained in this experiment are compared with the work done by de Swaaf *et al.* (2003a) (Table 11.3). In the former experiment, carried in 2 l working capacity fermenter, agitation was increased to 1250 rpm, yet aeration was only 1 $l\ min^{-1}$. Besides, Glucanex (Novo Nordisk, Neumatt, Switzerland) enzymatic solution was implemented to the media (0.5 $g\ l^{-1}$) in order to reduce medium viscosity and improve O_2 transfer. Both the different aerating conditions and the smaller vessel used were responsible for dissolved O_2 being maintained above 30 % in the previous experiment. In the present work dissolved O_2 was maintained over 30 % using O_2-enriched air, but this gave no further improvement to the previous fermentation in terms of growth, lipid and DHA content, indicating that this was not a critical parameter of the culture. Accordingly, we concluded that strain ATCC 50060 used might be responsible for the increase in r_{DHA} obtained in the present fermentation.

The present results confirmed that ethanol fed-batch method is optimal for DHA production by *C. cohnii*, demonstrating the potential of ATCC 50060 strain, which attained a final biomass attained of 91 $g\ l^{-1}$ in 210 h of

fermentation. Accordingly, the final r_{DHA} was the highest so far reported for this microalga, 56 mg DHA l^{-1} h^{-1} (using 10 % inoculum). Further culture optimization should concentrate in other parameters than dissolved O_2 that was not critical at the conditions tested.

Acknowledgements

This work was supported by Tomás de Zárate Grant (Cabildo de Gran Canaria). We thank Colin Ratledge and Alistair Anderson for his help and assistance in this work.

References

Cheng, A.T.Y. (1998) How to make oxygen economical for fermentation. In: Pharmaceutical Ingredients Worldwide, *CPHI press*, Amsterdam, Netherlands, pp. 1–12.

de Swaaf, M.E., Pronk, J.T. and Sijtsma, L. (2003a) Fed-batch cultivation of the docosahexaenoic-acid-producing marine alga *Crypthecodinium cohnii* on ethanol. *Appl Microbiol Biotech*, 61: 40–43.

de Swaaf, M.E., Sijtsma, L. and Pronk, J.T. (2003b) High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohni*. *Biotech Bioeng*, 81: 666–672.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernández-Cruz, C. (2000) Recent advances in lipid nutrition in fish larvae. *Fish Physiol Biochem*, 22:97–107.

Izquierdo, M.S., Fernández-Palacios, H. and Tacon, A.G.J. (2001) Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture*, 197(1–4):25–42.

Lichtenstein, A.H. (2003) Dietary fat and cardiovascular disease risk: quantity or quality? *J Women Health Gen-B*, 12: 109–114.

Lopes da Silva, T., Mendes, A., Mendes, R.L., Calado, V., Alves, S.S., Vasconcelos, J.M.T. and Reis, A. (2006) Effect of n-dodecane on *Crypthecodinium cohnii* fermentations and DHA production. *J Ind Microbiol Biot*, 3(6):408–16.

Montero, D., Socorro, J., Tort, L., Caballero, M.J., Robaina, L.E., Vergara, J.M. and Izquierdo, M.S. (2004) Glomerulonephritis and immunosuppres-

sion associated with dietary essential fatty acid deficiency in gilthead sea bream, *Sparus aurata* L., juveniles. *J Fish Dis*, 27(5):297–306.

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

Ratledge, C. (2002) Micro-organisms as sources of polyunsaturated fatty acids. In: Gunstone, F.D. (Ed), Structured and modified lipids, *Marcel Dekker Inc.*, Basel, New York, pp. 351–400.

Rodríguez-Ruiz, J., Belarbi, E.H., Sánchez, J.L.G. and Alonso, D.L. (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotech Tech*, 12(9):689–691.

Roux, M.P., Kock, J.L.F., Du Preez, J.C. and Botha, A. (1995) The influence of dissolved oxygen tension on the production of cocoa butter equivalents and gamma-linolenic acid by *Mucor circinelloides*. *Syst Appl Microbiol*, 18:329–334.

Sargent, J.R. and Tacon, A.G.J. (1999) Development of farmed fish: a nutritionally necessary alternative to meat. *P Nutr Soc*, 58(2):377–383.

Sijtsma, L., Springer, J., Meesters, P.A.E.P., de Swaaf, M.E. and Eggink, G. (1998) Recent advances in fatty acid synthesis in oleaginous yeast and microalgae. *Recent Res Dev Microbiol*, 2:219–232.

Sijtsma, L., Anderson, A.J. and Ratledge, C. (2005) Alternative carbon sources for heterotrophic production of DHA by the marine alga *Crypthecodinium cohnii*. In: Ratledge, C. and Cohen, Z. (Eds.) Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 107–123.

Terry, P.D., Rohan, T.E. and Wolk, A. (2003) Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. *Am J Clin Nutr* 77: 532–543.

Wynn, J., Behrens, P., Sundararajan, A., Hansen, J. and Apt, K. (2005) Production of single cell oils by dinoflagellates. In: Ratledge, C. and Cohen, Z. (Eds.) Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 86–98.

Chapter 8

Crypthecodinium cohnii and *Schizochytrium* sp. as potential substitutes to fisheries-derived oils in seabream (*Sparus aurata*) microdiets

This work was made at the Aquaculture Research Group (GIA) facilities (ULPGC & ICCM) and published in: Ganuza, E., Benítez-Santana, T., Atalah, E., Vega-Orellana, O., Ganga, R. and Izquierdo, M.S.(in press) *Aquaculture*.

Abstract

Two single cell heterotrophs were produced as alternative sources of docosahexaenoic acid (DHA; 22:6 n-3) to fisheries-derived oils. *Schizochytrium* G13/2S or *Crypthecodinium cohnii* biomasses, either homogenised (S_H, C_H, respectively) or non-homogenised (S, C), were tested in gilthead seabream (*Sparus aurata*) microdiets. In Trial 1 the biomases of both species of algae were used in replacement of fish oil, whereas in Trial 2 all dietary lipids, including those derived from squid meal, were replaced by the algal biomass in all diets except for control diet. Larval dietary acceptance, overall survival, air-exposure stress survival, growth and disease resistance were not significantly affected by the source of DHA employed when only fish oil was substituted. However, complete substitution of all dietary lipids by the algal biomass, resulted in reduced growth and survival which was apparently related to other dietary imbalances besides simply DHA content, such as changes in the DHA: EPA (eicosapentaenoic acid; 20:5 n-3) ratio. After 15 and 21 *d* feeding the experimental diets to *Sparus aurata* larvae in Trial 1 (0.1 *mg* initial larval dry weight) and Trial 2 (0.15 *mg* initial larval dry weight), respectively, the algal biomass inclusion or homogenization did not significantly affect the DHA deposition in fish body (14.4 ± 0.3 % total fatty acids ±SEM and 28.4 ±0.4 %, average values for Trial 1 and 2, respectively). Higher arachidonic acid levels were found in larvae fed *Schizochytrium* sp. diets in Trial 2

and were related to the retro-conversion from docosapentaenoic acid (DPA; 22:5 n-6) which was high in these diets. These results shows the potential of single cell heterotrophs as alternative DHA sources for fish oil in microdiets for gilthead seabream but also point out the necessity of EPA sources to completely replace fisheries derived oils.

Introduction

Fish oil has been the traditional source of highly unsaturated fatty acids (HUFA) in fish diets, essential nutrients for marine aquaculture species. Complete replacement of fish oil by vegetable oils, may cause unwanted secondary effects in fish health (Montero *et al.*, 2003) and flesh quality (Izquierdo, 2005) due to the lack of HUFA, such as docosahexaenoic acid (DHA; 22:6 n-3), in plant oils. Thus, the essential HUFA must be included in aquafeeds in order to ensure the production and nutritional quality of farmed fish. Fermentation technology permits us to step back in the food chain and produce alternative HUFA from microalgae through their heterotrophic cultivation. DHA is heterothrophically produced in high concentrations by the dinoflagelate *Crypthecodinium cohnii* and by the thraustochytrid *Schizochytrium* sp. Their cultivation is carried out under controlled sterile conditions where lipid accumulation can be tailored (de Swaaf *et al.*, 2003; Ganuza and Izquierdo, 2007). Therefore, these microalgae can supply a constant-quality product for aquafeeds that is not limited by nature. Despite that single cell heterotrophs have been successfully used as nutritional enrichment (Harel *et al.*, 2002), these HUFA sources seem also very promising as fish oil replacers in aquafeeds (Carter *et al.*, 2003; Miller *et al.*, 2007). In particular, fish oil substitution by *Crypthecodinium cohnii* in early weaning diets for gilthead seabream (*Sparus aurata*) improved fish survival as well as growth performance, in agreement with a higher proportion of DHA in diets and in total lipids of fish (Atalah *et al.*, 2007).

Indeed, although it has recently been found that the expression of genes involved in synthesis of these fatty acids is regulated by dietary lipids (Izquierdo *et al.*, 2007), *Sparus aurata* requires the inclusion of HUFA. HUFA requirements are particularly critical during the early life stages, as fish larvae need to maintain a fast growth (Izquierdo, 2005). Besides, larvae have a particularly high specific requirement for DHA, which is incorporated into visual and neural tissues (Mourente, 2003). A DHA deficiency in seabream is related to low growth and survival (Izquierdo, 1996), impaired predator behavior (Benítez-Santana *et al.*, 2007), skeletal deformities (Cahu *et al.*, 2003) and immune-suppression (Montero *et al.*, 2004). Hence, seabream larvae constitute an excellent nutritional model to study the dietary substitution of fish

oil by DHA-containing single cell heterotrophs.

The aim of the present study was to evaluate the potential of *Crypthecodinium cohnii* and *Schizochytrium* G13/2S to replace fisheries-derived lipids as a source of DHA in seabream microdiets. This work, which includes the production of alternative single cell sources, is proposed as a first step towards the implementation of sustainable diets that could contribute to maintain a renewable aquaculture supply, independent from fisheries resources.

Materials and Methods

Single cell heterotrophs The algae biomass was cultivated in a 10 l fermenter (Bioflo 3000, New Brunswick). *C. cohnii* ATCC 50060 biomass was produced according to the ethanol fed-batch method (de Swaaf *et al.*, 2003). *Schizochytrium* G13/2S (BNA 40-004), was produced according to the NH_4/pH-auxostat method (Ganuza *et al.*, in press). Both algal biomasses were harvested at the stationary phase of the culture by centrifugation (3,000 g, 5 min, 10 °C). The biomass was washed twice with demineralised water at 3 °C in order to eliminate the residual medium.

Experimental microdiets Five microdiets based on squid meal as the protein source were tested. Fish oil (Trial 1) and also squid meal lipids (Trial 2) from the control diet (FO) were substituted by *Schizochytrium* sp. and *C. cohnii* freeze-dried biomass, either as whole cell biomass (S, C, respectively) or homogenised biomass (S_H, C_H). Homogenisation was attained by a single pass through a French press (at 10 $Kpsi$ and -20 °C) in order to test the effect of the cell wall in larval DHA assimilation. All ingredients (Table 8.1) were pulverized in a Cyclotec 1093 sample mill (Rose Scientific Ltd., Stockholm, Sweden) and mixed in a mortar. Afterwards, they were mixed with gelatine dissolved in hot water (80 °C). The paste was pelleted with a pipping bag and dried in an oven at 40 °C for 24 h and then ground in the mill and sieved to obtain particle size below 200 μm.

In each trial, all experimental diets contained similar amounts of lipids, proteins (Table 8.1) and DHA (Table 8.2), however algal diets from Trial 2 contained slightly lower protein and lipid contents due to the displacement by the carbohydrate introduced by the algal biomass supplementation. The control diet (FO) in Trials 1 and 2 contained a heterogeneous fatty acid profile with similar DHA quantities but higher contents of arachidonic acid (ARA; 20:4 n-6) and eicosapentaenoic acid (EPA; 20:5 n-3) than those present in diets containing algal biomass, which, in turn, showed a high content of oleic acid (OA; 18:1 n-9) provided by olive oil (Table 8.2). The diets containing single cell heterotrophs reflected the fatty acid profile of the different algal

Table 8.1: Formulation and proximate composition of the experimental microdiets containing single cell heterotrophs.

	Trial 1[a]					Trial 2[b]				
	FO	S_H	*S*	C_H	*C*	*FO*	S_H	*S*	C_H	*C*
Ingredients (g 100 g^{-1} *of diet*)										
Sardine oil[c]	5.5	-	-	-	-	5.5	-	-	-	-
Olive oil	-	4.5	4.5	4.5	4.5	-	5.3	5.8	6.4	6.4
Squid meal[d]	74.5	73.5	73.5	73	73	-	-	-	-	-
Defatted squid meal[e]	-	-	-	-	-	65.8	55.5	53.7	59.6	59.6
Squid lipids[e]	-	-	-	-	-	8.7	-	-	-	-
Schizochytrium homogenised biomass (S_H)	-	2.5	-	-	-	-	21.4	-	-	-
Schizochytrium whole-cell biomass (*S*)	-	-	2.5	-	-	-	-	21.2	-	-
Crypthecodinium homogenised biomass (C_H)	-	-	-	2	-	-	-	-	15.6	-
Crypthecodinium whole-cell biomass (*C*)	-	-	-	-	2	-	-	-	-	15.6
Soybean lecithin	2	2	2	2	2	2	1.8	1.9	1.8	1.8
Attractant[f]	3	3	3	3	3	3	2.7	2.9	2.8	2.8
Vitamin mixture[g]	6	6	6	6	6	6	5.3	5.8	5.5	5.5
Mineral mixture[h]	4.5	4.5	4.5	4.5	4.5	4.5	4	4.3	4.1	4.1
Taurine	1.5	1.5	1.5	1.5	1.5	1.5	1.3	1.4	1.4	1.4
Gelatin	3	3	3	3	3	3	2.7	2.9	2.8	2.8
Proximate analysis[i] (% *dry weight*)										
Crude lipids	18	17	18	18	18	14	14	14	14	14
Crude protein	71	71	70	71	71	71	61	62	63	64
Ash	7	7	7	7	7	8	7	7	7	7

[a] Fish oil dietary-lipid substitution. [b]Fish oil and squid meal dietary-lipid substitution. [c]Agramar S.A., Spain. [d]Rieber & Son, Bergen, Norway. [e]Obtained after 3 extractions with chloroform. [f](Kanazawa *et al.*, 1989). [g]The vitamin mixture contained (mg 100 g $diet^{-1}$): Choline chloride (2965.8), inositol (1450.9), nicotinic acid (290.16), ascorbyl polyphosphate (180), α-tocopherol (150), p-aminobenzoic acid (145), calcium panthotenate (101.59), riboflavin (72.53), thiamin HCl (21.77), piridoxyne HCl (17.28), menadione (17.28), astaxanthin (5) cholecalciferol (3.65), retinol acetate (0.18), cyanocobalamin (0.03). [h](Teshima *et al.*, 1982). [i]Mean values (n=3) are quoted in order to include the analytical error.

species. Diets *C* and C_H contained higher 14:0 and 18:1 but lower 16:0 than *S* and S_H diets. Moreover, *Schizochytrium*-made diets contained docosapentaenoic acid (DPA, 22:5 n-6), a characteristic fatty acid produced by this organism. The higher DHA: EPA and DHA: ARA ratio of *FO* diets in comparison to algal diets was more autstanding in Trial 2 than in Trial 1 due to the higher degree of substitution.

Sparus aurata cultures Sixteen (Trial 1) and nineteen (Trial 2) day-old gilthead seabream (*S. aurata*) larvae of 5.13 $\pm$0.06 mm standard length ($\pm$SEM), 0.1 $\pm$0.00 mg dry body weight and 6.03 $\pm$0.09 mm, 0.15 $\pm$0.003 mg respectively, were manually transferred to 15 fibreglass cylindrical tanks (1900 larvae tank^{-1}) containing 170 l working volume. UV-sterilized water at

Table 8.2: Fatty acid composition of rotifers and microdiets containing fish oil (FO), *Schizochytrium* sp. homogenised biomass (S_h) or whole cell biomass (S) and *Crypthecodinium cohnii* homogenised biomass (C_h) or whole cell biomass (C).

Fatty acid[a]		Diets from Trial 1[b]					Diets from Trial 2[c]				
(%)	*Rotifers*	*FO*	S_H	S	C_H	C	*FO*	S_H	S	C_H	C
12:0	35.9	n.d.	n.d	n.d	n.d	n.d.	0.6	0.1	0.2	0.5	0.6
14:0	3.3	5.1	1.0	1.4	2.6	3.2	4.6	1.1	3.0	7.4	8.1
15:0	3.1	0.5	0.2	0.2	0.2	0.7	0.9	0.4	0.2	0.1	n.d
16:0 iso	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d	n.d	n.d.	n.d.
16:0	1.4	24.6	17.9	21.9	17.4	18.2	20.4	34.9	40.1	26.6	27.2
16:1 n–9	2.5	5.5	0.4	0.4	0.4	0.5	5.6	0.5	0.5	0.1	0.5
16:1 n–7	15.0	0.3	0.1	0.1	0.1	0.1	0.3	0.2	n.d.	n.d	n.d.
17:0	0.6	0.8	0.1	0.1	0.1	0.1	1.2	0.1	n.d.	n.d.	0.1
16:3 n–4	0.4	0.4	0.1	n.d.	0.3	0.3	1.2	n.d.	0.1	0.1	0.1
16:4 n–3	0.4	n.d.	n.d.	n.d.	n.d.	n.d.	0.4	n.d.	0.1	n.d.	n.d.
16:4 n–1	0.9	0.4	0.4	0.3	0.3	0.3	n.d.	n.d.	n.d.	n.d.	n.d.
18:0	1.7	4.2	2.5	2.4	2.8	2.6	4.0	3.4	2.4	3.9	3.7
18:1 n–9	20.7	8.0	37.7	36.2	37.0	38.3	7.4	28.7	27.1	34.7	34.4
18:1 n–7	2.7	2.3	1.3	1.3	1.4	1.5	2.5	n.d.	n.d.	n.d.	1.0
18:2 n–9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
18:2 n–6	1.5	9.9	7.0	6.6	7.3	7.0	9.2	6.8	5.2	8.0	7.4
18:3 n–6	0.4	0.1	n.d.	n.d.	n.d.	n.d.	2.0	0.1	n.d.	n.d.	n.d.
18:3 n–3	1.6	0.2	1.0	1.0	1.0	1.0	1.5	0.9	0.7	1.0	0.9
20:0	n.d.	0.2	0.1	0.1	0.1	0.1	0.7	0.4	0.2	0.4	0.3
20:1 n–9	2.9	n.d.	n.d.	n.d.	n.d.	n.d	2.4	0.3	0.2	0.4	0.3
20:3 n-3	0.3	0.9	0.6	0.5	0.5	0.5	0.2	n.d.	n.d.	n.d.	n.d.
20:4 n-6	n.d.	3.0	2.2	2.0	2.2	2.2	1.8	0.4	0.2	0.1	0.1
20:4 n-3	0.4	0.5	0.1	0.1	0.1	0.3	1.5	0.2	0.2	n.d	n.d.
20:5 n-3	n.d.	9.3	5.8	5.1	5.5	5.4	10.4	1.0	0.5	0.8	0.7
22:1 n–11	1.8	0.5	0.1	0.1	0.2	0.1	0.7	0.1	n.d.	0.3	n.d.
22:5 n–6	n.d.	0.3	1.0	0.9	0.1	0.2	n.d.	3.6	2.7	0.1	n.d.
22:5 n–3	n.d.	1.0	0.3	0.2	0.2	0.2	1.4	0.1	0.1	0.1	0.3
22:6 n–3	n.d.	16.3	18.2	17.0	16.6	16.2	16.4	15.4	15.2	14.9	14.0
Other FA	1.4	5.9	2.1	2.3	3.6	0.9	2.6	1.3	1.2	0.6	0.2
Saturates	46.1	35.5	21.8	26.0	23.1	24.9	32.5	40.3	46.0	38.8	40.2
Monounsaturated	46.5	17.0	39.9	38.3	39.5	40.9	18.6	29.6	27.8	35.5	36.2
$(n-3)$ HUFA	0.0	27.0	24.4	22.4	22.4	22.0	29.7	16.8	16.0	15.8	14.9
$(n-3)$: $(n-6)$	10:10	10:5	10:4	10:4	10:4	10:4	10:4	10:6	10:5	10:5	10:5
DHA: EPA	0:0	10:6	10:3	10:3	10:3	10:3	10:6	10:1	10:0.3	10:1	10:0.5
DHA: ARA	0:0	10:2	10:1	10:1	10:1	10:1	10:1	10:0.3	10:0.1	10:0.1	10:0.1
$(n-3)$ HUFA: OA	0:21	10:3	10:15	10:16	10:17	10:17	10:2	10:17	10:17	10:22	10:23

[a] The values (n=1) are quoted in order to include the analytical error. [b]Fish oil dietary-lipid substitution.[c]Fish oil and squid meal-lipid substitution.

20–22 °C was provided at 250-300 $ml\ min^{-1}$ into the tank and the overflow filtered through a 300 mm mesh to remove non-ingested food. Air flow was maintained constant at 250 $ml\ min^{-1}$. During the 12 h photoperiod (1500 lux) microdiet was manually spread into the surface every 45 min (2 g day^{-1}) and during the first 10 d of the experiment yeast-fed rotifers (1.5 ind. ml^{-1}) were supplied twice, at noon and mid afternoon, in a co-feeding regime. Larvae were fed during 15 (Trial 1) and 21 (Trial 2) days. The tank and the water surface were cleaned and siphoned daily. All treatments were performed in randomly distributed triplicates.

After 7 d of treatment the larvae from Trial 1 were challenged by the parasite *Amylodinium ocellatum*, an algal protozoan (Dinoflagellida) that affects seabream hatcheries producing white opaque protuberances in skin and gills, which eventually causes larval mortality. The parasite was inoculated from previously contaminated nets by locating them in the water reservoir that flows into the tanks. Parasite affectation was determined by counting the number of lateral protuberances present in 20 larvae tank^{-1}.

Dietary acceptance was calculated from the micrographs (25 X) of 20 larvae per tank taken at the end of each experiment. The gut area, as well as the area occupied by the diet was calculated through the transparent larvae with Image-Pro Plus 6.0 software. Survival was determined by manually counting live larvae at the beginning and end of each experiment. Larval resistance to stress was determined at the end of each experiment through an air-exposure stress test of 20 larvae maintained for 1 min on a scoop net. Following the stress, larvae were moved to a 2 l aerated container in which survival was manually calculated 24 h later. Larval growth was determined by measuring at the beginning, middle and end of the trial, the standard length of 20 larvae per tank with a profile projector (Nikon V-12, Nikon co., Tokio, Japan). These larvae were then washed in distilled water and dried in an oven (110 °C) until constant weight, in order to measure the mean larval dry weight. A sample of initial and all surviving larvae were washed in distilled water, drained and deposited in plastic bags and stored at -80 °C until proximal composition analysis.

Biochemical analyses Dietary and larval moisture, protein and lipid content were determined according to the Association of Official Analytical Chemists (AOAC) described methods (A.O.A.C., 1995). Fatty acid methyl esters were obtained by transesterification with 1 % sulphuric acid in methanol (Christie, 2003) and then separated by gas chromatography (GC; Thermo Finnigan) with He as a carrier gas using a fused silica, carbowax 20M, 30 m x 0.32 mm i.d. (df = 0.27 m) column (Supelco, Bellefonte, USA). The initial

temperature of the column was set to 170 °C for 10 *min*, then it was raised to 220 °C at 2.5 °C min^{-1} and finally maintained at 215 °C for a further 5 *min*. The temperature of the injection port and flame-ionisation port was 250 °C. The peaks in the chromatogram were identified with well-characterised external standards (Sigma).

Statistical analyses Results were compared in Sigmastat 3.5 software. One-way ANOVA and Tukey test for multiple mean comparison were applied after passing normality and equal variance test. When variances were not homogeneous, means were compared by Kruskal-Wallis test. Significance was accepted at probabilities of 0.05 or less.

Results

Single cell heterotrophs The fermentation time of *Schizochytrium* G13/2S was shorter (49 *h*) than that of *C. cohnii* (192 *h*). According to the fast growth observed in *Schizochytrium* sp., the overall DHA productivities were 125 *mg* DHA l^{-1} h^{-1}, while *C. cohnii* produced only 56 *mg* DHA l^{-1} h^{-1}. Probably as a consequence of the fast growth, *Schizochytrium* G3/2S showed a lesser incidence of unwanted microbial contamination than occurred in various *C. cohnii* fermentations that were subsequently discarded.

***Sparus aurata* performance** All diets were equally well accepted by larvae regardless of the DHA source employed. Only during the parasite infection in Trial 1, a decrease in the ingestion of all diets was found in terms of % of gut area occupied by the diet (9.5 ±1.3 %), in comparison to the healthy larvae from Trial 2 (46.8 ±3.2 %) that were bigger with 40 day of development. The overall larval survival and the survival to the air exposure stress test in Trial 1 was not affected by dietary inclusion of algal biomass (Figure 8.1). However, in Trial 2, complete dietary lipids substitution by the algal biomass significantly decreased overall survival and survival to air exposure stress test in comparison to the larvae fed *FO* diet. There were no significant differences between the performance of larvae fed with *Schizochytrium* sp. and *C. cohnii*, either homogenised or non homogenized, except for a slightly lower, but significant survival in larvae fed homogenized *Schizochytrium* sp. in Trial 2. Fish oil substitution by the algal biomass in Trial 1 did not significantly affect growth (standard length and total body weight) of *S. aurata* (Figure 8.2). However, in Trial 2, complete substitution of dietary lipids in algal diets markedly reduced larval growth in comparison with *FO* diet. Nine days after challenging larvae from Trial 1 to the ectoparasite *A. ocellatum*, the characteristic white nodules were observed on

the surface of the seabream larvae. There were no significant differences in the parasite incidence between the dietary sources, the average count on the larvae lateral body surface remaining 18 ±2 nodules larvae^{-1}.

The larvae protein and lipid contents were not affected (P>0.05) by dietary treatments, being respectively 78.5 ± 0.7 and 15 ± 0.2 % dry weight in Trial 1 and 72 ±1 % and 21 ±0.4 % in Trial 2. The DHA content in larvae was similar in all treatments regardless of the lipid source (fish oil or algae biomass) and of the processing of algal biomass employed (homogenised or non homogenised) (Table 8.3). However, the fatty acid profile did reflect that of the lipid sources employed. Thus, profiles of *FO* fed larvae showed more peaks than those from algal diets. *FO* larvae contained less OA but higher EPA than larvae fed with algal biomass, reflecting the lower EPA content in microbial sources. Accordingly, a major difference in fatty acid composition of larvae fed with algal diets was the higher DHA: EPA ratio.This was especially significant in Trial 2, in relation with a lower dietary DHA:EPA ratio in Trial 2 (complete lipid substitution) than in Trial 1. Although ARA was initially present in low quantities in the algal diets, the larval content of this fatty acid was not affected by inclusion of algal biomass in Trial 1. In Trial 2, in comparison with *FO* larvae, ARA content was significantly lower in *C. cohnii*-fed larvae, but higher in those fed *Schizochytrium*, coinciding with the incorporation of the characteristic 22:5 n-6. The n-3/n-6 ratio in diets was lower than in larval body in all Trials.

Discussion

Two single cell heterotrophic strains were tested as dietary lipid sources for larval seabream, due to their capacity to produce DHA, an essential fatty acid frequently obtained from fish oil. The cultures of both *Schizochytrium* G13/2S and *C. cohnii* ATCC 50060 were previously optimised through the implementation of several medium and fermentation technologies (de Swaaf *et al.*, 2003; Ganuza *et al.*, in press). In the present study, the fermentation of *Schizochytrium* sp. was more stable and productive than fermentation of *C. cohnii*. The whole-cell biomass produced by these heterotrophs was high in DHA and low in carbohydrate and ash. These characteristics encourage their utilization in fish diets, enabling manipulation of the dietary fatty acid content according to the particular nutritional requirements of each fish species. Taking into account that the bulk fish oil contains higher levels of EPA than DHA, single cell heterotroph enrichment might be very advantageous in certain aquaculture applications with higher requirements for DHA. For instance, fish larvae possess higher specific requirements for DHA (Izquierdo, 1996), which is difficult to concentrate from fish oil due to

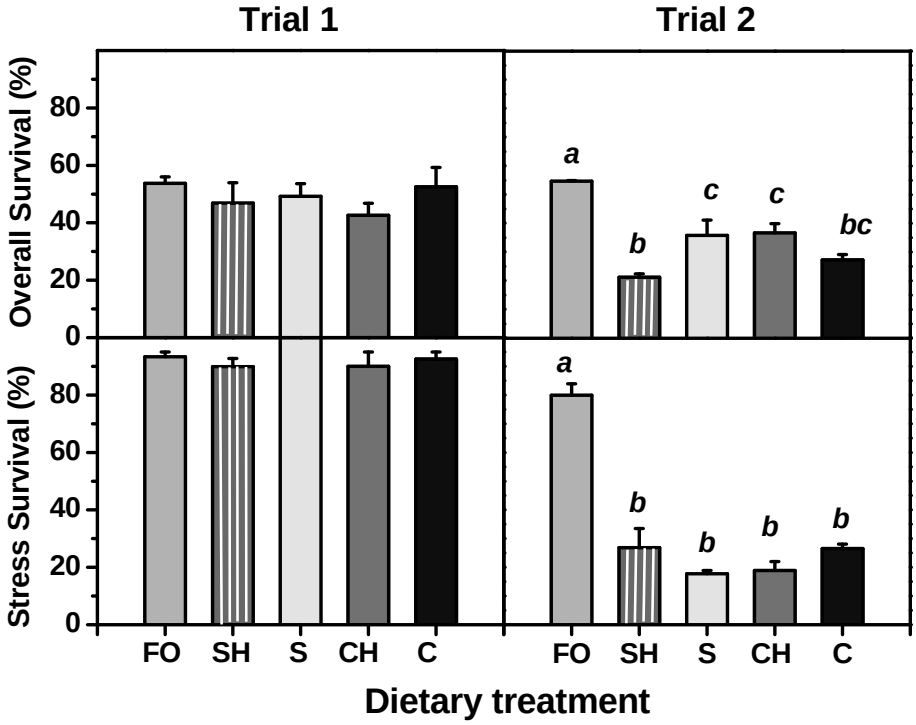


Figure 8.1: Overall survival and air-exposure stress test survival of *Sparus aurata* larvae fed with microdiets containing alternative DHA sources: either *Schizochytrium* G13/2S homogenised biomass (S_h), whole cell biomass (S), *Crypthecodinium cohnii* homogenised biomass (C_h) or whole cell biomass (C) were used as DHA substitute to fisheries-derived lipids. In Trial 1 (from 16 to 31 day post hatch) the lipids provided by fish oil in control diet (FO) were fully replaced, while in Trial 2 (from 19 to 40 day post hatch) both the lipids provided by fish oil and the lipids from the protein source were fully replaced. Each column represents the average (n=3) of three replicate cultures quoted with the correspondent error bars (± SEM). Columns containing different letters are significantly different ($P<0.05$).

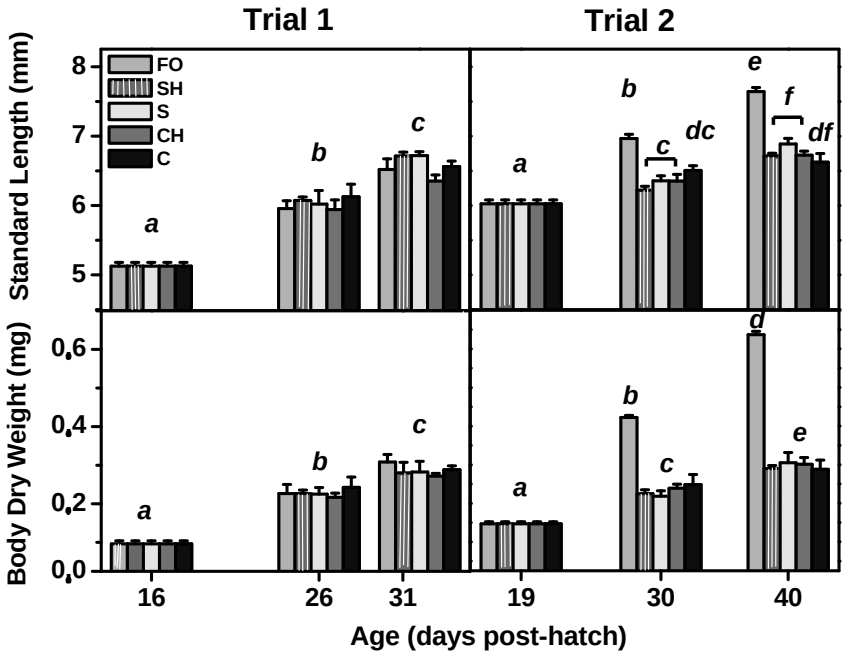


Figure 8.2: Growth of *Sparus aurata* larvae fed with microdiets containing alternative DHA sources according to Figure 8.1 methodology.

Table 8.3: Fatty acid composition of *Sparus aurata* larvae fed with microdiets containing fish oil (FO), *Schizochytrium* sp. homogenised biomass (S_h) or whole cell biomass (S) and *Crypthecodinium cohnii* homogenised biomass (C_h) or whole cell biomass (C).

Fatty acid[a]	Larvae from Trial 1[b]						Larvae from Trial 2[c]					
(%)	*Initial*	*FO*	S_H	*S*	C_H	*C*	*Initial*	*FO*	S_H	*S*	C_H	*C*
12:0	n.d	n.d.	n.d	n.d	n.d	n.d.	n.d	0.3	1.3	0.6	0.6	0.5
14:0	0.6	n.d.	0.1	0.0	0.1	0.6	0.8	1.4	1.2	1.3	1.4	1.7
15:0	0.6	0.0	0.3	0.3	0.5	0.8	0.4	0.4	0.6	0.6	0.4	0.3
16:0 iso	0.7	0.7	0.4	0.4	0.5	0.5	0.6	0.4	0.1	0.4	0.4	0.0
16:0	15.8	26.2	21.8	23.7	23.1	21.5	11.3	21.1	17.1	19.4	15.0	16.1
16:1 n–9	6.3	4.6	3.8	3.0	3.7	3.4	4.8	3.1	1.7	0.9	0.9	10.9
16:1 n–7	0.9	0.5	0.5	0.4	0.5	0.5	0.6	0.4	0.9	1.2	1.3	1.3
17:0	0.3	0.7	0.5	0.3	0.7	0.4	1.2	0.6	0.4	0.8	0.5	0.4
16:3 n–4	0.9	0.9	0.6	0.6	0.4	0.4	0.9	0.5	0.5	0.3	0.7	0.8
16:4 n–3	0.1	1.0	0.5	0.4	0.6	0.7	0.6	0.4	0.2	0.5	0.7	0.6
16:4 n–1	0.5	0.4	0.5	0.4	0.4	0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:0	10.4	10.6	10.4	9.4	10.0	9.3	8.3	8.6	7.1	7.8	6.6	7.0
18:1 n—9	15.9^a	12.8^a	24.9^b	25.5^b	22.8^b	24.4^b	13.9^a	8.7^b	18.6^c	18.7^c	23.2^d	22.5^d
18:1 n–7	3.8	3.7	3.7	3.1	3.4	3.0	4.5	2.7	2.3	2.0	2.4	1.9
18:2 n–9	1.4	0.8	1.1	0.8	1.0	0.9	1.9	0.2	n.d.	0.1	0.1	n.d.
18:2 n–6	5.0	4.9	3.8	4.2	3.3	4.3	8.7	5.7	5.6	5.5	6.2	6.3
18:3 n–6	0.3	0.1	0.1	0.1	0.2	0.2	0.4	0.2	0.2	0.1	0.2	0.2
18:3 n–3	1.4	0.3	0.1	0.3	0.2	0.3	0.2	0.6	0.9	0.9	0.8	0.9
20:0	0.9	1.6	1.2	1.2	1.1	0.8	3.0	0.3	0.2	0.1	0.1	0.3
20:1 n–9	1.2	0.3	1.0	0.9	1.0	1.7	2.1	1.3	0.7	0.4	0.6	0.8
20:3 n-3	0.2	0.1	0.1	0.1	0.1	0.1	0.4	1.0	0.2	0.3	0.4	0.3
20:4 n-6	1.9^a	1.3^b	1.1^b	0.9^b	0.9^b	1.0^b	2.0^a	1.6^b	2.2^a	1.9^a	1.1^c	1.2^c
20:4 n-3	0.6	0.1	n.d	0.1	0.1	0.1	0.6	0.3	0.1	0.1	0.1	0.2
20:5 n-3	4.1^a	4.4^a	2.6^b	2.9^b	2.7^b	2.8^b	6.1^a	8.0^a	2.6^b	1.8^b	2.6^b	2.6^b
22:1 n–11	0.3	0.4	0.1	0.4	0.4	0.3	0.2	0.2	0.2	0.1	0.1	0.2
22:5 n–6	0.5^a	0.3^a	1.0^b	0.7^b	0.3^a	0.2^a	n.d.	n.d.	4.2^a	4.4^a	0.0^b	0.2^b
22:5 n–3	1.7	0.7	0.4	0.3	0.3	0.5	2.2	1.9	0.8	0.4	0.8	0.8
22:6 n–3	15.6^a	14.2^a	13.9^a	14.1^a	14.2^a	15.4^a	21.6^a	28.5^b	26.3^b	28.5^b	29.8^b	28.9^b
Other FA	8.0	8.4	5.5	5.5	7.8	5.6	3.0	1.5	2.9	1.1	3.0	3.0
Saturated	28.6	39.2	34.3	35.0	35.5	33.5	24.9	32.7	27.9	30.5	24.7	26.2
Monounsaturated	28.9^a	22.7^b	34.5^c	33.6^c	32.1^c	33.7^c	25.5^a	16.1^b	23.4^a	22.1^a	27.2^a	26.4^a
$(n-3)$ HUFA	22.0^a	19.4^b	17.0^b	17.3^b	17.2^b	18.7^b	30.4^a	38.6^b	30.9^a	30.8^a	33.2^a	32.4^a
$(n-3)$: $(n-6)$	10:3	10:3	10:3	10:3	10:3	10:3	10:4	10:2	10:4	10:4	10:2	10:2
DHA: EPA	10:3	10:3	10:2	10:2	10:2	10:2	10:3	10:3	10:1	10:1	10:1	10:1
DHA: ARA	10:1	10:1	10:1	10:1	10:1	10:1	10:1	10:1	10:1	10:1	10:0.4	10:0.4
$(n-3)$ HUFA: OA	10:7	10:7	10:15	10:15	10:13	10:13	10:5	10:2	10:6	10:6	10:7	10:7

[a] Each figure represents the average of three replicate cultures containing an error (± SEM) < 10 % of its value. Different treatments containing a different superscript, in Trial 1 or Trial 2, are significantly different (P<0.05). n.d.: not detectable.[b]Fish oil dietary-lipid substitution.[c]Fish oil and squid meal-lipid substitution.

the abundance of other polyunsaturated fatty acids.

Inclusion of the two mentioned single cell heterotrophs in *S. aurata* microdiets resulted in the larvae attaining the same levels of DHA as those obtained by feeding fish oil, denoting the high nutritional value of the algae biomass as an alternative source of this fatty acid. Algae biomass, as a complex source of micro and macronutrients, showed no antigrowth (Francis *et al.*, 2001) neither a beneficial effect on health (Verschuere *et al.*, 2000; Rodríguez *et al.*, 2003) above the pure nutritional DHA supplementation effect. Thus, the inclusion of algal biomass did not affect parasite resistance in the challenge test, despite the higher mortality described in Atlantic salmon pre-smolt challenged against *Vibrio anguillarum* when DHA was completely substituted in diet by a thraustochytrid (Carter *et al.*, 2003). There were no differences in the DHA incorporation level attained with whole-cell and homogenised cell biomass, indicating the adecuate nutritional utilization of this fatty acid by *S. aurata* larvae, at this stage of development, even when the algal cell wall remains intact. If confirmed in other species and stages of development, these results might have positive implications in the industrial fermentation since the homogenisation step could be eliminated from the downstream process. AfFurther advantage of using the DHA rich whole-cell biomass is the natural encapsulation, since this protects the valuable fatty acids from exposure to oxidative agents.

The direct inclusion of single cell heterotrophs in microdiets, rather than the laborious implementation as live prey enrichment, allows a better-defined study of fish larvae lipid nutrition, without interference of live prey metabolism to the analysis. Due to the few fatty acids present in microbial lipids used in this study, fatty acid consumption and deposition were examined through the complete substitution of dietary fish oil. The single cell biomass containing microdiets are low in the essential EPA. Although live prey are usually able to obtain this fatty acid from the oxidation of DHA from microbial sources (Barclay and Zeller, 1996; Ritar *et al.*, 2004), the *S. aurata* larvae fed with algal diets showed a very low incorporation of EPA, indicating that both oxidation from DHA or synthesis through Δ^6 and Δ^5 desaturation (Tocher and Ghioni, 1999) to produce EPA was not performing, at least sufficiently, in this species. Although the n-3 HUFA content attained in the larval body was appropriate (2.5 % body dry weight), EPA requirements, which at this stage should be half DHA requirements (Rodríguez *et al.*, 1998), were not fulfilled in Trial 2 (DHA: EPA 10:1). Due to the role of EPA as one the most important precursor of eicosanoids, its deficiency in tissue might affect the cell immune and the stress responses (Ganga *et al.*, 2005, 2006). Therefore, even when the DHA requirements are fulfilled in the diet, EPA deficiencies could hamper growth, survival and resistance to stress (Liu *et al.*, 2002) as ob-

served in Trial 2. Similarly, ARA levels in algal diets were low in the present study, but this fatty acid was incorporated in larval lipids above the dietary levels as also described in turbot (Linares and Henderson, 1991). Moreover, both ARA and DPA contents in larvae fed *Schizochytrium*-containing diets were higher than in fish oil or *C. cohnii*-fed larvae, suggesting that DPA was retro-converted to ARA by oxidation, either by the rotifers or by the larvae, as pointed out by Sargent *et al.* (1997). Thus, beyond the interest of *Schizochytrium* as a DHA source, this alga might also contribute indirectly with ARA. Despite the small quantities in which ARA is usually present in fish, this is an important precursor of a different family of eicosanoids which interacts with EPA derivatives regulating the response of many physiological processes (Bell and Sargent, 2003), influencing larval growth, survival (Bessonart *et al.*, 1999) and stress response (Koven *et al.*, 2001; Van Anholt *et al.*, 2004). Since EPA rather than ARA levels in larval lipids correlated with the differences in larval performance in Trial 2, the effect of ARA was probably masked by that of EPA as pointed out by Bessonart *et al.* (1999). Therefore, EPA deficiency, along with other factors in the algae-containing diet in Trial 2, such as differences in the lipid, protein or vitamins contents, might contribute to the low larval performance in this study.

The optimum DHA:EPA ratio (Rodríguez *et al.*, 1998) can be provided when only fish oil is substituted in microdiets because the squid meal is maintained as a source of EPA. Alternatively, use of DHA single cell sources as live prey enrichment can also provide adequate DHA: EPA ratios, since both *Artemia* and rotifers are able to retro-convert the former (Evjemo *et al.*, 2001; Ritar *et al.*, 2004), thus increasing EPA levels in *S. aurata* fish larvae (Koven *et al.*, 2001; Harel *et al.*, 2002). However, fish oil replacement by algal biomass, despite providing an excellent DHA source for marine fish larvae, did not allow the complete substitution of fisheries products in larval diets, due to the low EPA content in larvae, indicating that single cell or other EPA sources are also required in order to provide larvae with an optimun DHA: EPA ratio. Microalgae such as *Odontella aurita*, *Monodus subterraneus* or *Nitzschia laevis* as well as fungi (*Mortierella alpina*) contain high quantities of EPA (Wen and Chen, 2005). However, the cultivation of these organisms must be further developed in order to attain the productivities attained with DHA-producing *Schizochytrium*. The capacity to develop heterotrophic technologies might reduce the production costs from more than US $ 20 kg^{-1} in phototrophic cultures to less than US $ 5 kg^{-1} (Gladue and Maxey, 1994) and eventually allow a economically feasible industrial production. Meanwhile, the microalgae assayed here can effectively replace fish oil as a source of DHA in diets for larvae and also for fingerlings of seabream (Atalah *et al.*, 2007) and Atlantic salmon parr (Miller *et al.*, 2007). Its implementa-

tion in less demanding fish feeding stages, such as juveniles or pre-adults, will depend on the production cost and its competitiveness with fish-based products.

In conclusion, whole, single cell heterotrophs were effectively used as DHA sources in *S. aurata* microdiets. For this purpose, *Schizochytrium* sp., due to its fast growth in fermenters and its capacity to increase ARA levels in fish larvae, is preferred to *C. cohnii*. Nevertheless, in order to provide larvae with the optimum DHA: EPA ratio, in the absence of fisheries-derived oils, efforts must be focused on the feasible production and testing of EPA sources.

Acknowledgements

We thank Colin Ratledge and Alistair J. Anderson for their collaboration in the fermentation work. This work was partially supported by the Toms de Zrate Grant (Cabildo de Gran Canaria).

References

A.O.A.C., 2007. Official methods of analysis of the Association of Analytical Chemist. U.S.A.,1018–1035.

Atalah, E., Hernández-Cruz, C.M., Izquierdo, M.S., Rosenlund, G., Caballero, M.J., Valencia, A. and Robaina, L. (2007) Two microalgae *Crypthecodinium cohnii* and *Phaeodactylum tricornutum* as alternative source of essential fatty acids in starter feeds for seabream (*Sparus aurata*). *Aquaculture*, 270(1–4):178–185.

Barclay, W. and Zeller, S. (1996) Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. *J World Aquacult Soc*, 27(3):314–322.

Bell, J.G. and Sargent, J.R. (2003) Arachidonic acid in aquaculture feeds: current status and future opportunities. *Aquaculture*, 218(1):491–499.

Benítez-Santana, T., Masuda, R., Juárez-Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M. and Izquierdo, M.S. (2007) Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. *Aquaculture*, 264(1–4):408–417.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M. and Fernández-Palacios, H. (1999) Effect of dietary arachidonic acid levels on growth and survival of gilthead seabream (*Sparus aurata*) larvae. *Aquaculture*, 127(1–4):265–275.

Cahu, C.L., Zambonino-Infante, J.L. and Takeuchi, T. (2003) Nutritional components affecting skeletal development in fish larvae. *Aquaculture*, 227(1–4):254–258.

Carter, C.G., Bransden, M.P., Lewis, T.E. and Nichols, P.D. (2003) Potential of thraustochytrids to partially replace fish oil in Atlantic salmon feeds. *Mar Biotechnol*, 5:480–492.

Christie, W.W. (2003) Lipid analysis. *Pergamon*, Oxford, UK, 3^{th} edition.

de Swaaf, M.E., Pronk, J.T. and Sijtsma, L. (2003) Fed-batch cultivation of the docosahexaenoic-acid-producing marine alga *Crypthecodinium cohnii* on ethanol. *Appl Microbiol Biot*, 61(1):40–43.

Evjemo, J.O., Danielsen, T.L. and Olsen, Y. (2001) Losses of lipid, protein and n-3 fatty acids in enriched *Artemia franciscana* starved at different temperatures. *Aquaculture*, 193(1–2):65–80.

FAO (2007) The state of world fisheries and aquaculture 2006. FAO Technical report, Fisheries Department, Rome, Italy.

Francis, G., Makkar, H.P.S. and Becker, K. (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199:197–227.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S. (2005) Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). *Comp Biochem Physiol B*, 142(4):410–418.

Ganga, R., Tort, L., Acerete, L., Montero, D. and Izquierdo, M.S. (2006) Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*. *J Endocrinol*, 190:39–45.

Ganuza, E., Anderson, A.J. and Ratledge, C. (in press) High-cell-density cultivation of *Schizochytrium* in ammonium-pH-auxostat fed-batch system. *Biotechnol Lett*.

Ganuza, E. and Izquierdo, M.S. (2007) Lipid accumulation in *Schizochytrium* G13/2S produced in continuous culture. *Appl Microbiol Biotech*, 76(5):985–990.

Gladue, R.M. and Maxey, J.E. (1994) Microalgal feeds for aquaculture. *J Appl Phycol*, 6(6):131–141.

Harel, M., Koven, W., Lein, I., Bar, Y., Behrens, P., Stubblefield, J., Zohar, Y. and Place, A.R. (2002) Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs. *Aquaculture*, 213(2):347–362.

Izquierdo, M.S. (1996) Essential fatty acid requirements of cultured marine fish larvae. *Aquacult Nutr*, 2(2):183–191.

Izquierdo, M.S. (2005) Essential fatty acid requirements in Mediterranean fish species. *Cah Options Mediterr*, 63:91–102.

Izquierdo, M.S., Robaina, L., Juárez-Carrillo E., Oliva, V., Hernández-Cruz, C.M. and Afonso J.M. (in press) Regulation of growth, fatty acid composition and Δ^6-desaturase expression by dietary lipids in gilthead seabream larvae (*Sparus aurata*). *J Fish Physiol.*

Kanazawa, A., Koshio, S. and Teshima, S. (1989) Growth and survival of larval red seabream *Pagrus major* and japanese flounder *Paralichthys olivaceus* fed microbound diets. *J World Aquacult Soc*, 20:31–37.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P. and Tandler, A. (2001) The effect of dietary arachidonic acid (20:4 n-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. *Aquaculture*, 193(1–2):107–122.

Linares, F. and Henderson, R.J. (1991) Incorporation of 14C-labelled polyunsaturated fatty acids by juvenile turbot, *Scophthalmus maximus* (L.) *in vivo*. *J Fish Biol*, 38:335–347.

Liu, J., Caballero, M.J., Izquierdo, M.S., El-Sayed-Ali, T., Hernández-Cruz, C.M., Valencia, A. and Fernández-Palacios, H. (2002) Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead seabream *Sparus aurata*. *Fisheries Sci*, 68(6):1165–1172.

Miller, M.R., Nichols, P.D. and Carter, C.G. (2007) Replacement of fish oil with thraustochytrid *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. *Comp Biochem Physiol A*, 148, 382–392.

Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M.J. and Izquierdo, M.S. (2003) Vegetable lipid sources for seabream (*Sparus aurata*): effects on fish health. *Aquaculture*, 225:353-370.

Montero, D., Socorro, J., Tort, L., Caballero, M.J., Robaina, L.E., Vergara, J.M. and Izquierdo, M.S. (2004) Glomerulonephritis and immunosuppression associated with dietary essential fatty acid deficiency in gilthead seabream, *Sparus aurata* L., juveniles. *J Fish Dis*, 27(5):297–306.

Mourente, G. (2003) Accumulation of DHA (docosahexaenoic acid; 22:6n-3) in larval and juvenile fish brain. In: Browman, H.I. and Skiftesvik, A.B., (Eds), *The Big Fish Bang*, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, Bergen, Norway, pp. 139–148.

Ritar, A.J., Dunstan, G.A., Nelson, M.M., Brown, M.R., Nichols, P.D., Thomas, C.W., Smith, E.G., Crear, B.J. and Kolkovski, S. (2004) Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. *Aquaculture*, 239(1–4):351–373.

Rodríguez, A., Esteban, M.A. and Meseguer, J. (2003) A mannose-receptor is possibly involved in the phagocytosis of *Saccharomyces cerevisiae* by seabream (*Sparus aurata* L.) leucocytes. *Fish Shellfish Immu*, 14(5):375–388.

Rodríguez, C., Pérez, J.A., Badía, P., Izquierdo, M.S., Fernández-Palacios, H. and Hernández, A.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(1–2):9–23.

Sargent, J.R., Mc Envoy, L.A. and Bell, J.G. (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture*, 155(1):117–127.

Teshima, S., Kanazawa, A. and Sakamoto, S. (1982) Microparticulate diets for the larvae of aquatic animals. *Data File Fish Res Kagoshima Univ*, 2:67–86.

Tocher, D.R. and Ghioni, C. (1999) Fatty acid metabolism in marine fish: low activity of Δ^5 desaturation in gilthead seabream (*Sparus aurata*) cells. *Lipids*, 34(5):433–440.

Van Anholt, R.D., Spanings, F. A.T., Koven, W.M., Nixon, O. and Wendelaar Bonga, S.E. (2004) Arachidonic acid reduces the stress response of gilthead seabream *Sparus aurata* L. *J Exp Biol*, 207(19):3419–3430.

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol R*, 64(4):655–671.

Wen, Z. and Chen, F. (2005) Prospects for eicosapentaenoic acid production using microorganisms. In: Ratledge, C. and Cohen, Z. (Eds.), Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 138–181.

Chapter 9

General conclusions

1. As demonstrated under chemostat steady state conditions, lipid accumulation in *Schizochytrium* sp. can be induced by decreasing nitrogen source concentration while carbon source is in excess.

2. *Schizochytrium* sp. accumulates some storage lipids (22 % CDW) even under carbon growth-limiting conditions, a peculiarity not yet described in other oleaginous microorganisms. This will respond to a more sophisticated type of "K-strategy" behavior, instead of the expected reproductive response.

3. *Schizochytrium* sp. possesses a growth-associated pattern of lipid accumulation that confers it the potential to produce docosahexaenoic acid in continuous culture. Therefore, lipid production is not necessarily a bi-phasic process as usually described for the lipid accumulating microorganisms.

4. The air supply has no influence in the docosahexaenoic acid (22:6 n-3; DHA) percentage of the lipids from *Crypthecodinium cohnii* or *Schizochytrium* sp., suggesting that this fatty acid might be synthesised by an alternative O_2-independent route in both organisms.

5. Odd-chain fatty acid production influences DHA accumulation in *Schizochytrium* and can be restricted by avoiding propionate precursors in the nitrogen source employed. For this purpose, a defined mineral medium, containing no sources of propionic acid, was developed for growing this organism.

6. Optimum DHA productivities in *Schizochytrium* G13/2S and *Crypthecodinium cohnii* are attained in an ammonium/pH-auxostat and an ethanol fed-batch systems respectively. The continuous feeding of the batch culture, responding to the organism requirements (as these systems are implemented), is an appropriate way of increasing growth in both organisms. *Schizochytrium* G13/2S is at least twice more productive than *C. cohnii*.

7. Docosahexaenoic acid incorporation from microbial sources in *Sparus aurata* larvae is equivalent to that of the traditional fish oil sources. Besides, no differences were found between the two microbial species. Further to that, the homogenisation treatment did not improve assimilation or culture performance in larvae.

8. Docosapentaenoic acid from *Schizochytrium* sp. can increase the levels of the essential arachidonic acid in *Sparus aurata* larvae by retroconversion during the culture, after the co-feeding regime.

9. Microbial sources can successfully substitute fish oil in *Sparus aurata* microdiets but whole dietary lipid substitution alerts about the EPA deficiency in larval body. Thus, the present work points out the necessity of also prospecting EPA sources in order to attempt fish oil complete substitution.

Chapter 10

Summary

10.1. Summary

This thesis focuses on the development of new microbial sources of docosahexaenoic acid (22:6 n-3; DHA) and their implementation in aquafeeds. The supply of highly unsaturated fatty acids is one of the major challenges to be faced by the aquaculture industry, as it still depends on the fish oil obtained from wild fisheries to produce their feeds. Thus, the aim of the research is to develop and optimise a sustainable process integrating the production of this essential fatty acid from heterotrophic microorganisms and its implementation in fish microdiets. For this purpose, two marine microalgae, *Schizochytrium* G13/2S (Thraustochytrid) and *Crypthecodinium cohnii* (Dinoflagellida), were cultivated in fermenters under controlled conditions. In order to identify the relevant parameters of the proces for the production of DHA, special attention was paid to the study of the lipid accumulation physiology and biochemistry. Accordingly, several cultivation protocols were developed, analysed and optimised with respect to the production of biomass, lipid and DHA. The nutritional properties of the algal biomass in fish diet were investigated in seabream (*Sparus aurata*) larvae due to their well known requirements for DHA. The nutritional performance upon fish oil replacement was evaluated in larvae dietary acceptance, growth, survival, stress tolerance and disease resistance, paying special attention on the fatty acid transformations and accumulation within the body.

Chapter 3 describes how *Schizochytrium* G13/2S, coinciding with its capacity to store an intracellular nitrogen pool, was able to grow and accumulate lipids simultaneously. Under continuous culture, lipid accumulation was promoted with low nitrogen concentrations while maintaining glucose at saturating levels. In turn, carbon limiting conditions in continuous culture did not impede lipid accumulation as expected from other oleaginous microorganisms. These characteristics showed that continuous culture, due to the good DHA productivities, as compared to those obtained in batch culture, probes encouraging for industrial consideration. In addition, the medium employed in continuous and fed-batch cultures inhibited the synthesis of 15:0

and 17:0 fatty acids, on behalf of other fatty acids (Chapter 5). This was associated to the lack of propionic acid precursors in glutamate or ammonium. On the other hand, the air supply improved growth and lipid accumulation in *Schizochytrium* G13/2S and *C. cohnii* but DHA proportion in fatty acid was not affected, suggesting that O_2, often considered as the limiting nutrient in industrial fermentations, is not involved in the desaturation steps synthesising this fatty acid (Chapter 4). To conclude the microbial work, various fermentation strategies were developed for both organisms. Finally, optimum DHA productivities in *Schizochytrium* G13/2S and *C. cohnii* were attained in an ammonium/pH-auxostat (Chapter 6) and an ethanol (Chapter 7) fed-batch systems respectively. *Schizochytrium* G13/2S was at least twice productive than *C. cohnii*.

Chapter 8 focuses on the effect of fish oil replacement by both algae (either homogenised or as a whole cell) in microparticulate diets for seabream. DHA incorporation by larvae was equivalent in all treatments indicating that microbial oils, even as a whole cell biomass, were good sources of DHA. Moreover, *Schizochytrium* provided n-6 docosapentaenoic acid which might contribute to the increasing levels of the essential arachidonic acid by retroconversion. Dietary intake, growth, survival, stress response and disease resistance were not compromised by the dietary substitution of fish oil, however, the larvae growth performance decreased when both fish oil and lipids from the protein source in microdiets were substituted, suggesting that microbial eicosapentaenoic acid sources should also be implemented.

10.2. Resumen

Esta tesis se centra en el desarrollo de nuevas fuentes microbianas de ácido docosahexaenóico (22:6 n-3; DHA) y su implementación en piensos para acuicultura. El suministro de ácidos grasos altamente insaturados es uno de los grandes retos a los que se enfrenta el sector acuícola, ya que hoy en día aún depende del aceite extraído de las pesquerías silvestres para confeccionar sus piensos. El objetivo de esta investigación es el de desarrollar y optimizar un proceso sostenible que integre la producción de este ácido graso a partir de microorganismos heterótrofos y su implementación en microdietas para peces. Con este fin, se cultivaron en fermentadores, bajo condiciones controladas, dos microalgas marinas: *Schizochytrium* G13/2S (Thraustochytrid) and *Crypthecodinium cohnii* (Dinoflagellida). Se identificaron los parámetros más relevantes para la producción de DHA a partir del estudio de la bioquímica y la fisiología de la acumulación lipídica. En consecuencia se desarrollaron, se analizaron y optimizaron varios protocolos para la producción de biomasa, lípidos y DHA. Las propiedades nutricionales de la biomasa algal como

alimento para peces fueron investigadas en las larvas de dorada (*Sparus aurata*) debido a los altos requerimientos de DHA que éstas poseen. Se evaluó la sustitución del aceite de pescado de la dieta en términos de aceptación, crecimiento, supervivencia, tolerancia al estrés y resistencia a las enfermedades, siguiendo como modelo la asimilación y transformación de los ácidos grasos dentro del cuerpo.

El Capítulo 3 describe cómo el *Schizochytrium* G13/2S, coincidiendo con su capacidad para guardar reservas de nitrógeno intracelular, es capaz de crecer y acumular lípidos simultáneamente. En cultivo continuo, bajo saturación de glucosa, las bajas concentraciones de la fuente de nitrógeno promueven la acumulación de lípidos. Las concentraciones limitantes de carbono, al contrario de lo esperado en otros organismos oleaginosos, permiten la acumulación de lípidos. Estas características observadas durante el cultivo continuo sugieren, en relación a las buenas productividades de DHA alcanzadas en comparación al cultivo tipo "batch", que éste puede ser susceptible de interés industrial. Además, el medio usado durante el cultivo continuo y también el cultivo "fed-batch" inhiben la síntesis de 15:0 and 17:0 en beneficio del resto de los ácidos grasos presentes en el *Schizochytrium*. La inhibición parece estar relacionada con la ausencia de precursores de ácido propiónico en el ácido glutámico y el amonio (Capítulo 5). Por otro lado, el suministro de aire al cultivo mejoró el crecimiento y la acumulación de lípidos en *Schizochytrium* G13/2S y *C. cohnii* pero la proporción de DHA en los ácidos grasos se mantuvo constante, sugiriendo que el O_2, considerado el factor limitante durante las fermentaciones a nivel industrial, no está implicado en la cadena de desaturaciones que conforma este ácido graso (Capítulo 4). Se desarrollaron varias estrategias de fermentación para ambos organismos. Finalmente, las mejores productividades de DHA obtenidas en *Schizochytrium* G13/2S y *C. cohnii* se alcanzaron en un pH-auxostat de amonio (Capítulo 6) y en un "fed-batch" de etanol (Capítulo 7) respectivamente. El *Schizochytrium* G13/2S fue al menos dos veces más productivo que *C. cohnii*.

El Capítulo 8 se centra en el efecto de la sustitución del DHA del aceite de pescado por las microalgas (tanto homogeneizadas como sin homogeneizar) en dietas microparticuladas para la dorada. La incorporación del DHA en las larvas fue equivalente en todos los tratamientos, indicando que las fuentes microbianas de DHA, sin necesidad de homogeneización, son adecuadas para un correcto aporte de este ácido graso a la larva. Además, el *Schizochytrium* presenta ácido n-6 docosapentaen'oico, el cual parece contribuir por retroconversión a los niveles del ácido araquidónico. La aceptación de las dietas, el crecimiento, la supervivencia, la respuesta al estrés y la resistencia a las enfermendades no se vio afectada por la sustitución del aceite de pescado en las microdietas, sin embargo, el crecimiento larvario disminuyó cuando el

aceite de pescado, así como los lípidos de la fuente proteica, fueron sustituidos, indicando la necesidad de implementar fuentes microbianas de ácido eicosapentaenóico.

10.3. Laburpena

Tesis hau docohexaenoic gantz-azidoaren (22:6 n-3; DHA) sorburu berri baten ikerketan oinarritzen da. Esate baterako mikrobioengandik ekoitzia eta azken batean arrain pentsuetan erabiltzeko. Gaur egun akuikulturaren pentsuak arrain olioa edukitzen duenez, sectorearen fio haundientakoa gantz-azido saturbetegabeen ordezko horniketa dauka. Horretarako ikerketa honen objektiboa, DHA-gan antolatutako microbioen hazkuntza eta mikrobio honen arrain nutrizio jarbidea, prozesu berberan baterabiltzea, gehitzea eta hobetzea da. Mikrobioak fermentadoreetan kontrolatutak hazi ziren, bi itsas mikroalga erabili ziren: *Schizochytrium* G13/2S (Thraustochytrid) eta *Crypthecodinium cohnii* (Dinoflagellida). Mikrobioen DHA-ren ekoitziarentzako parametro garrantzitsuenak bildu ziren, koipetasun procesuaren biokimika eta fisiolojiaren ikasbidez. Informazio hauek erabili ziren mikroalgaren biomasa, koipetasuna eta DHA eikoizpenaren protokoloak gehitzeko eta obetzeko. Urraburuaren (*Sparus aurata*) larbak ereduz artuz, DHA-rengan behar haundia daukatenez, mikroalga biomasaren nutrizio ezaugarriak ikertu ziren. Arrain olioaren ordezko microalgen jarrera saiatu zen. Horretarako, Urraburuaren pentsuaren onarpena, larven haziketa, biziraupena, estresaren jarrera eta gaixotasunaren erresistentzia ikertu ziren. Parametro guzti hauek azaldu ziren larben gantz-azidoen transformazio eta asimilazioari begiraz.

3-arren Kapituloan azaltzen da nolatan *Schizochytrium* G13/2S, microbio honen barne nitrogeno erreserba biltzeko ezaugarriarengatik, hazi eta aldiberezko koipetasunari molda daiteke. Kemostatoan, glucosa ase eta nitrogeno konzentrazio moteletan, koipetasuna bultzatzen dute. Carbon konzentrazio motelak edo murristutak, beste koipedun microbioen alderantziz, ez dute koipetazuna mugatzen. Kemostatoan behatutako ezaugarri ahuek, esate baterako DHA emankortasun onak, "batch"-enaren emankortasunari parekatuta, ernarazten dute kemostatoaren industrial baliagarritasunaren garrantzia. Horrez gain, microbioak kemostatoan edo "fed-batch"-en medioan, *Schizochytrium*-en 15:0 and 17:0 gantz-azidoren sintesia eragozten du, beste gantz azidoen honerako. Badirudi, inhibizio hau propioniko azidoaren faltarengandik sortuta dagoela (5-garren Kapituloa). Bestalde, kultivoaren aireberritzea *Schizochytrium* G13/2S eta *C. cohnii*-ren haziketa eta koipetasuna hobetzen du. Hala ere, DHA-aren proporzioa ez da aldatzen. Hau adierazten du O_2-ak (aunitz induztrial fermentazioaren mugatzailea) ez duela parte hartzen gantz azido honen sintesian (4-garren Kapituloa). Fermentazio estrategi be-

rriak gehitu ziren bi microalgentzako. Azkenik, *Schizochytrium* G13/2S eta *C. cohnii*-en DHA emankortasun hoberenak, amonio"pH-auxostat" batean (6-garren Kapituloan) eta etanol "fed-batch" batean (7-ren kapituloan) gainditu ziren urrenez urren. *Schizochytrium* G13/2S izan zen gutzienez bi aldiz *C. cohnii* baino DHA-ren emankorrago.

8-garren Kapituloa mikroalgen (homojenizatua edo homojenizatugabea), arrain olioaren ordezkapenaren ondorioetan oinarritzen da. Naiz eta pentsu bat edo beste erabili, urraburuaren larben DHA assimilazioa berbera izan zen. HAu adierazten du: mikrobo DHA-ak, bai homogenizatua bai homogenizatugabea, arrain olioaren DHA-aren alternativa bikaina da. Bestalde, *Schizochytrium*, n-6 dokosapentaenoic gantz-azidoa daukanez, molekula hau retrokonversio bidez geitu dezake arakidonik gantz-azidoari. Arrai olioaren ordezkapena, ez zuen aldatu: urraburuaren pentsuaren onarpena eta larven hazkundea, biziraupena, estresaren jarrera eta gaixotasunaren erresistentzia. Ala ere, bai arrain olioa bai pentsuaren lipido guztiak ordezkatu zirenean, larben haziketa moteldu zen, eicosapentaenoic gantz-azidoaren mikrobio sorburuaren garranzia zakontzen.

Capítulo 11

Resumen ampliado

11.1. Introducción general (1)

Los lípidos y su función biológica

Los lípidos constituyen un grupo de compuestos orgánicos que son insolubles en agua y solubles en solventes orgánicos. A pesar de que no existe una definición estructural que englobe a todos los lípidos, muchos de ellos, los saponificables, están constituidos por ácidos grasos: una cadena molecular hidrocarbonada que en uno de sus extremos tiene un grupo carboxilo y en el otro un grupo metilo (el carbono n u ω). La cadena hidrocarbonada puede estar saturada o insaturada. Los ácidos grasos más frecuentes en los seres vivos poseen una cadena par de entre 12 y 24 carbonos con entre 0 y 6 insaturaciones o dobles enlaces intercalados por un grupo metilo.

Dado que los lípidos son un grupo de compuestos muy heterogéneo y variado, existen diferentes clasificaciones. Por ejemplo, en función de su polaridad, los lípidos se dividen entre polares y neutros. Los últimos incluyen ácidos grasos y sus derivados como glicerolípidos, esteroles, ceras y tocoferoles, entre otros. Dentro de los lípidos polares se encuentran los glicerofosfolípidos (también llamados fosfolípidos), glicoglicerolípidos, esfingomielina y glicoesfingolípidos.

Los ácidos grasos poliinsaturados (PUFA) tienen entre 18 y 24 carbonos y dos o más instauraciones en su cadena alifática. Los PUFA más frecuentes en la naturaleza son: el ácido docosahexaenóico (DHA; 22:6 n-3) (Figure 1.1), docosapentaenóico (DPA; 22:5 n-3 o n-6), eicosapentaenóico (EPA; 20:5 n-3), araquidónico (ARA; 20:4 n-6), γ-linolénico (GLA; 18:3 n-6), α-linolénico (LNA; 18:3 n-3) y linoléico (LA; 18:2 n-6). Los ácidos grasos altamente insaturados (HUFA), entre los que se incluyen el DHA, DPA, EPA y ARA, son PUFA con 20 ó 22 átomos de carbono en su cadena alifática y con 3 ó más dobles enlaces.

Los ácidos grasos conforman la estructura de las membranas celulares y, por lo tanto, están presentes en todas las formas de vida. Son los responsables de recibir la información y anticipar una respuesta ante cualquier estrés que

perturbe la organización intracelular. Los cambios en la composición de los ácidos grasos pueden alterar radicalmente el funcionamiento de la membrana. Por lo tanto, su papel no es tan sólo estructural o energético, sino también funcional, ya que toman parte en diversos procesos como el control de la permeabilidad y plasticidad de la membrana, la división celular, la formación de hormonas o la respuesta inmune.

La fluidez de la membrana depende fuertemente de las condiciones ambientales, así como de su composición interna. Cualquier variación ambiental puede afectar a la fluidez de la membrana, lo que actuará como receptor canalizando la respuesta reguladora de la célula (Beney and Gervais, 2001). La célula puede alterar el contenido en ácidos grasos insaturados de los fosfolípidos de membrana y restablecer así su fluidez. Debido a la estructura tridimensional que confieren las instauraciones a la cadena alifática, las interacciones hidrógeno-hidrógeno entre moléculas adyacentes disminuyen, mejorando la fluidez y la inserción proteica en la membrana. Por ello, el índice de instauración de *Escherichia coli* aumenta al disminuir la temperatura de cultivo, manteniendo así una viscosidad óptima en la membrana (Nakayama *et al.*, 1980). El contenido en PUFA mejora la tolerancia al estrés, la supervivencia al calor y al etanol (Peyou-Ndi *et al.*, 2000), la adaptación osmótica a la salinidad (Khaware *et al.*, 1995) o a la congelación (Giraud *et al.*, 2000). En particular, el DHA posee un estructura muy resistente a los cambios de temperatura y presión en la membrana (Rabinovich and Ripatti, 1991) y modula la disposición de las cadenas alifáticas, su fluidez, cambio de fase, compresibilidad elástica, permeabilidad, movimientos flip-flop y actividad proteica (Stillwell and Wassall, 2003). En organismos superiores el DHA, facilita los rápidos cambios en la conformación de las proteínas de membrana que están asociados a procesos visuales y neuromusculares (Brown, 1994).

Además del papel de los fosfolípidos de membrana como medio para la inserción de los receptores ambientales, canales iónicos y enzimas también actúan como reservorio de mensajeros entre células. Los PUFA actúan como precursores de potentes mediadores bioactivos implicados en la respuesta celular y en la comunicación entre células (Kingsella *et al.*, 1990). Estas moléculas señalizadoras tienen una gran repercusión en la regulación de la respuesta inmune (Calder *et al.*, 2002), la coagulación, la respuesta inflamatoria y la homeostasis celular (Calder, 2002), así como en la transmisión y plasticidad de la sinapsis, la neuroprotección y la regulación del ciclo del sueño (Chen and Bazan, 2005). El ácido dihomogammalinolénico (DGLA; 20:3ñ-6, araquidónico (ARA; 20:4ñ-6) y eicosapentaenóico (EPA; 20:5ñ-3) se liberan de los fosfolípidos de membrana mediante la acción de la fosfolipasa A2 (Kingsella *et al.*, 1990). Como ácidos grasos libres, compiten por el mismo sitio activo en la enzima ciclo-oxigenasa y la lipooxigenasa para la producción

de sus correspondientes eicosanoides, una serie de hormonas entre las que se incluyen las prostaglandinas, tromboxanos y leucotrienos, entre otros. De forma similar, el DHA compite por estas enzimas para producir resolvinas y protectinas, que también están asociadas al control inflamatorio (Hong *et al.*, 2003; Serhan *et al.*, 2002). Por ello, el perfil de ácidos grasos de los fosfolípidos de membrana modula el tipo de respuesta metabólica del organismo. La deficiencia de ciertos ácidos grasos en la dieta o el desequilibrio entre el ratio de los ácidos grasos de las series n-3/n-6 puede producir estados patológicos asociados a la supresión inmune o a procesos inflamatorios tales como: enfermedades auto-inmunes, artritis reumática, la enfermedad de Crohn, cáncer de pecho, de cólon, de próstata, enfermedades coronarias, ataques al corazón, subdesarrollo retinal y cerebral durante la infancia (Connor, 2000), así como enfermedades de tipo neurológico como la epilepsia y el Alzheimer (Chen and Bazan, 2005).

Los lípidos neutros se usan también como forma de almacenamiento de carbono y energía. Estas moléculas hidrofóbicas forman agregados en agua que permiten un empaquetado compacto y eficiente. La energía almacenada de esta forma óptima, estaría disponible para su uso durante el ayuno de la célula. Muchos organismos, desde los microorganismos oleaginosos o las semillas, hasta peces como el salmón o la anguila, almacenan glicerolípidos antes de la reproducción o cualquier otro proceso ambiental o biológico adverso. Los ácidos grasos saturados y otros ácidos grasos insaturados de menos de 20 carbonos presentes en los glicerolípidos son fácilmente β-oxidados en la mitocondria para la obtención de la energía metabólica que se acumula en forma de ATP. El DHA, sin embargo, es pobre como sustrato para la β-oxidación mitocondrial, y generalmente es oxidada en el peroxisoma (Madsen *et al.*, 1999).

La bioquímica de los lípidos

La síntesis de ácidos grasos "*de novo*" se cataboliza por un complejo enzimático llamado ácido graso sintetasa (FAS), responsable de la elongación de la cadena alifática. Este proceso se inicia con la condensación entre malonato y el acetil co-A mediante el aporte de un ATP y es considerado el paso limitante de la reacción. La enzima 3-ketoacil reductasa con un aporte de NADPH introduce el extremo carboxilo en el C_3. La deshidratación entre el C_1 y el C_3 por parte de la encima 3-ketoacil reductasa y una molécula de NADPH produce un sustrato enoil que a continuación será reducido por la enzima enoil reductasa. En consecuencia la cadena inicial será alongada en dos carbonos. Por lo tanto, el ácido palmítico es el resultado de una secuencia de siete ciclos de elongación en el que el FAS utilizará acetil co-A como

unidad molecular. Este sistema determina la síntesis de los ácidos grasos en la mayoría de los organismos eucariotas. La ruta tradicionalmente aceptada para la síntesis de PUFA parte del ácido palmítico y sus derivados, y requiere una cascada de reacciones catalizadas por series alternadas de enzimas elongasas y reductasas (Figure 5.1(a)). El ácido linoléico (LA; 18:2 n-6), el ácido α-linolénico (ALA; 18:3 n-3) y el ácido oleico (18:1 n-9) son los precursores que inician la síntesis de los PUFA de las series n-6, n-3 y n-9, respectivamente. Estos sustratos compiten por la enzima Δ^6-desaturasa, la primera en la ruta de síntesis de PUFA. Estas desaturasas se llama "front-end" porque introducen la instauración cerca del extremo carboxilo de la cadena alifática (Δ^6 indica que el doble enlace se introduce a seis carbonos de distancia del carbono Δ o carboxílico). Las enzimas elongasas y desaturasas que le siguen también actuarán cerca del extremo carboxilo de la cadena y, como resultado, la posición de la primera instauración se conservará a lo largo de la ruta, formando las distintas familias de PUFA (n-3, n-6 y n-9). En general, las enzimas desaturasa tienen mayor afinidad por los sustratos n-6, seguidos de los n-3 y finalmente n-9. Ya que el LA puede interferir en la conversión de ALA y disminuir la tasa n-3/n-6, es muy importante el mantener una tasa adecuada entre las distintas familias de ácidos grasos durante la alimentación. Las desaturasas son enzimas que requieren O_2 para actuar y en su mayoría están ligadas a las membranas (Klein and Volkmann, 1975; Meyer and Bolch, 1963). Por tanto, esta ruta es la responsable de la síntesis de los ácidos grasos más comunes en la naturaleza, a la que tradicionalmente se le conoce como ruta desaturasa/elongasa y es una ruta aeróbica.

La secuencia de la ruta "desaturasa/elongasa" sugiere que el DHA es, en última instancia, el producto de la Δ^4 desaturasa (Qiu *et al.*, 2001). Sin embargo, esta enzima no ha sido aún aislada en mamíferos. En humanos, el DHA parece estar sintetizado mediante una extensión de la ruta tradicional que evita la acción de la Δ^4-desaturasase y se denomina "Ruta de Sprecher" (Sprecher, 2002; Voss *et al.*, 1991). Esta ruta comprende dos elongaciones consecutivas del EPA y la acción de la Δ^6-desaturasa producirían el 24:6ñ-3, que finalemte necesitaría de un ciclo de β-oxidación en el peroxisoma para producir el DHA sin mediación de la Δ^4-desaturasa. Por lo tanto esta ruta necesitaría de la migración del 24:6 n-3 desde el retículo endoplasmático al peroxisoma para su oxidación parcial. De forma equivalente, la extensión de la ruta de la serie n-6 produciría el DPA 22:5ñ-6 a partir del 22:4ñ-6 y sin la mediación de la Δ^4-desaturasa.

La nueva ruta de tipo bacteriano para la síntesis de DHA, la poliquetido sintasa (PKS), es radicalmente diferente a lo arriba establecido. PKS ha sido descrita en algunas bacterias (*Shewanella spp.* y *Moritella marina*), y al menos a un organismo eucariota (*Schizochytrium* sp.) (Metz et al., 2001). La

configuración genética para la síntesis de ácidos grasos que presentan estos organismos es aparentemente más sencilla que la tradicional. Mantiene cierto parecido al sistema FAS responsable de la síntesis del ácido palmítico; sin embargo, en PKS la cadena alifática se elonga y conserva la insaturación hasta la producir HUFA sin la mediación de las enzimas desaturasas (Figure 5.1(b)). Durante el ciclo de elongación, la cadena alifática no es reducida mediante la deshidratación, sino que actúa una isomerasa, convirtiendo la instauración de su configuración *trans* a *cis*. En consecuencia, las instauraciones se conservan sin necesidad de las enzimas desaturasas, lo cual supondrían un costo energético adicional de un NADPH para la reducción y otro para su posterior desaturación. Se trata, por tanto, de una ruta independiente al O_2 que introduce las instauraciones con una eficiencia energética mucho mayor (Ratledge, 2004).

Desde un punto de vista ecológico, la síntesis *de novo* de los PUFA está confinada a determinados nichos ecológicos, principalmente ecosistemas acuáticos. Los vegetales terrestres ofrecen una fuente alimentaria ácidos grasos esenciales de hasta 18-carbonos, pero los vertebrados, entre ellos la mayoría de los peces marinos, tienen una capacidad limitada de convertir estos ácidos grasos a HUFA de cadena mayor (Brenna, 2002). Con pocas excepciones, los productores primarios capaces de sintetizar HUFA *de novo* están restringidos al medio marino. Debido a la importancia biológica de estos ácidos grasos,se ha observado que muchas interacciones tróficas están muy relacionadas con su presencia (Masuda, 2003). Por ejemplo, los episodios evolutivos del *Homo sapiens* están relacionados con los ambientes acuáticos, en los cuales la presencia de HUFA en dieta ayudó a sostener el desarrollo neuronal (Broadhurst and Wang, 2002; Crawford *et al.*, 1999). Por otro lado, el flujo de carbono entre productores primarios y consumidores en los ecosistemas dulceacuícolas se pudo predecir en función del contenido HUFA de los niveles tróficos (Müller-Navarra *et al.*, 2004, 2000). En la naturaleza, la cadena alimentaria provee al pez de la mayoría de los HUFA esenciales que necesita (Ackman *et al.*, 1964). La acuicultura de peces y crustáceos marinos depende de los lípidos procesados por la industria pesquera para su alimentación ya que no es posible cosechar en el océano los productores primarios (fitoplanton) responsables de la producción de este nutriente.

Importancia de los HUFA en peces

Los primeros intentos para cultivar larvas de peces marinos con rotíferos chocaron con las altas mortalidades larvarias asociadas a la deficiencia de HUFA en la dieta (Watanabe *et al.*, 1983). Durante el desarrollo embrionario y larvario, antes de que comience la alimentación externa, los huevos o el saco

vitelino contribuyen con todos los nutrientes necesarios. Sin embargo, una vez que comienza la alimentación externa, el organismo debe sintetizar todos los compuestos que no son suministrados en la dieta a la velocidad que necesitan sus nuevos tejidos que en esta etapa experimentan un rápido crecimiento. Por ello, cuando la larva consume los nutrientes del saco vitelino, las deficiencias dietéticas de HUFA se manifiestan en altas mortalidades, mal crecimiento y baja actividad (Izquierdo, 1996, 2005). A la vista de la resistencia que presentan los huevos, en relación a otros tejidos, a modificar su composición a través de la dieta de los reproductores, se cree que su composición podría reflejar los requerimientos nutritivos de los estadios siguientes. De hecho, los huevos de peces marinos (Sargent *et al.*, 2003), en particular los de la dorada (*Sparus aurata*) (Mourente and Odriozola, 1990), contienen altas cantidades de HUFA en sus fosfolípidos. Además, durante el ayuno, las larvas de dorada tienden a conservar los n-3 HUFA en los fosfolípidos de membrana antes que otros ácidos grasos (Koven *et al.*, 1989) lo cual indica la importancia de estos nutrientes. Los primeros intentos de éxito en el cultivo de peces marinos utilizaron emulsiones de aceites de pescado ricos en n-3 HUFA con lecitina como enriquecedores de presas vivas (Watanabe *et al.*, 1983).

El DHA, EPA y el ARA son ácidos grasos esenciales para el crecimiento y desarrollo adecuado (Watanabe, 1982), así como para la reproducción de peces marinos (Izquierdo *et al.*, 2001). Al contrario que los peces de agua dulce, los peces marinos tienen una capacidad limitada, casi despreciable para transformar el LNA a EPA y el LA a ARA (Owen *et al.*, 1975; Tocher *et al.*, 2003). En la dorada, la tasa de conversión está principalmente limitada por la baja actividad de la Δ^5-desaturasa (Tocher and Ghioni, 1999). Por lo tanto, el pez obtiene los HUFA principalmente a través de la dieta que luego incorpora selectivamente en sus tejidos. La dieta debe tener unos niveles óptimos de HUFA pero, además, los distintos ácidos grasos esenciales, con sus correspondientes funciones metabólicas, deben ser incluidos en proporciones adecuadas, las cuales son diferentes para cada especie, estadio del desarrollo e incluso pueden variar en función de las condiciones metabólicas del organismo (smoltification) (Sargent and Tacon, 1999).

El estadio larvario en peces marinos tiene requerimientos específicos por el DHA mayores que en alevines y juveniles. Este ácido graso se incorpora en el tejido visual y neuronal (Mourente, 2003), que durante el desarrollo constituye una mayor proporción del peso corporal que en estadios más avanzados. Una dieta deficitaria en DHA esta asociada a la disminución de la habilidad de las larvas de arenque para ver y capturar sus presas (Bell *et al.*, 1995), al retraso en el desarrollo del comportamiento del medregal japonés (Masuda *et al.*, 1998) y la dorada (Benítez-Santana *et al.*, 2006), a la incidencia de deformidades esqueléticas en varias especies (Cahu *et al.*, 2003), a la inmu-

nosupresión (Montero *et al.*, 2004) y a la mala pigmentación y migración del ojo de los peces planos (Bell *et al.*, 2003; Mc Evoy *et al.*, 1998). El DHA está considerado superior como ácido graso esencial para el crecimiento, la resistencia al estrés en la dorada japonesa entre otras especies (Watanabe *et al.*, 1989; Watanabe and Kiron, 1994). Por ello es necesario controlar los niveles del EPA en dieta, puesto que este ácido graso compite con el DHA y puede desplazar su incorporación en las membranas celulares. El requerimiento de HUFA de las larvas de dorada (*Sparus aurata*), habitualmente del 1.5 % del peso seco (Rodríguez *et al.*, 1998), puede incrementar hasta cuatro veces su valor ante la preponderancia de EPA sobre DHA (Rodríguez *et al.*, 1994b). El ratio óptimo DHA: EPA en larvas es aproximadamente de 10: 5 (Rodríguez *et al.*, 1994a, 1997) pero en juveniles puede bajar a menos de 10: 10 (Ibeas *et al.*, 1997). En cuanto al ARA, al ser un HUFA menos abundante, ha recibido menos atención que otros ácidos grasos y, sin embargo, se trata, junto con el EPA (Ganga *et al.*, 2006), de uno de los más importantes precursores de eicosanoides. La implementación de ARA en la dieta contribuye a mejorar la respuesta al estrés y la supervivencia en larvas de dorada (Bessonart *et al.*, 1999; Van Anholt *et al.*, 2004), aunque estas mejoras no siempre son observables debido a la influencia del ratio DHA:EPA en la dieta (Ibeas *et al.*, 1997; Robin and Peron, 2004) u otros factores.

Los distintos niveles de interacción entre los ácidos grasos esenciales deben ser considerados a la hora de definir las necesidades dietéticas de las distintas especies. La primera interacción competitiva entre los ácidos grasos de la dieta ocurre a nivel digestivo. La lipasa activada de sal biliar (BAL) en la dorada japonesa (*Pargus major*) tiene una mayor afinidad por el EPA y el ARA que por el DHA de la dieta (Iijima *et al.*, 1998). Durante la absorción y el transporte, los fosfolípidos de la microdietas mejoran la incorporación de n-3 HUFA (en particular del DHA) en los lípidos polares de la dorada (Salhi *et al.*, 1995). Las acilasas y transacilasas, son responsables de esterificar los ácidos grasos a los distintos fosfolípidos y tienen diferentes afinidades en función del ácido graso que esterifiquen, interfiriendo así en el transporte e incorporación a los tejidos. Por ejemplo, el ARA está principalmente asociado a la segunda posición del glicerol del fosfatidilinositol (PI), mientras que el DHA se encuentra en la segunda posición de la fosfatidilcolina (PC) y la fosfatidiletanolamina (PE) (Sargent *et al.*, 2003). El DHA en la dieta compite con el EPA por la segunda posición del PE, por lo tanto un ratio alto de DHA/EPA en la dieta aumenta la esterificación del DHA al PE mejorando el crecimiento de la dorada (Izquierdo *et al.*, 2000). Además, el metabolismo de síntesis y catálisis de los HUFA puede seleccionar y modificar el perfil inicial que dieta induzca en el organismo. Generalmente, las enzimas desaturasa y elongasa se estimulan con su sustrato y se inhiben con su producto. Como

indicábamos anteriormente, la tasa de conversión de n-3 y n-6 PUFA a HUFA de cadena más larga está limitada por la baja actividad de ciertas enzimas de la ruta, tanto la Δ^5-desaturasa como la C_{18} a C_{20} elongasa (Tocher *et al.*, 2003). Por tanto, no solo el balance entre los precursores de las series n-3 y n-6, LA y ALA puede alterar la proporción de los HUFA (y sus derivados bioactivos) sino que, de acuerdo con la ruta de "Sprecher", descrita al menos en la trucha arcoiris (Buzzi *et al.*, 1997), tanto el LA y ALA, como el 24:4ñ-6 o el 24:5ñ-3 podrían ser sustratos en competencia por la misma Δ^6-desaturasa. Si bien se desconoce si actúa la misma enzima en C_{18} y C_{24}, esta posibilidad sugiere interacciones aún más complejas entre ácidos grasos. Por otro lado, los HUFA son oxidados en menor porporción que los ácidos grasos saturados o monoinsaturados, y se conservarían gracias al catabolismo diferencial. Igualmente, la oxidación diferencial de los distintos HUFA puede alterar su proporción en los tejidos. Por ejemplo, los lípidos del atún tienen una mayor concentración de DHA que de EPA en respuesta al catabolismo preferencial del EPA frente al DHA (el cual se oxida en el peroxisoma) (Sargent *et al.*, 2003). Por último, existe otro tipo de competición entre los HUFA a nivel fisiológico por la liberación de eicosanoides. El ARA y el EPA son sustratos de las lipoxigenasas que catalizan la producción de leucotrienos de las series 4 y 5 y de la ciclooxigenasa que cataliza la producción de prostaglandinas de la 2 y 3 series respectivamente (Kingsella *et al.*, 1990). Las moléculas señalizadoras compiten por los mismos receptores celulares, pudiendo dar respuestas antagonistas en función del ácido graso del que derivan (Whelan, 1996). Aunque habitualmente el ARA se encuentra presente en menor proporción que el EPA, el ARA posee una mayor afinidad por la ciclooxygenasa. El EPA en cambio tiene mayor afinidad por la lipooxigenasa. La respuesta fisiológica estará en última instancia determinada por los niveles relativos de ambos ácidos grasos en el PI. Debido al carácter complejo de las interacciones entre HUFA y a la posibilidad de interferencia competitiva entre ambos, la proporción en la que los tres principales ácidos grasos esenciales deben estar presentes en la dieta debería ser definida en ratios conjuntos además de en valores absolutos (Izquierdo, 1996; Sargent and Tacon, 1999; Izquierdo *et al.*, 2000).

Importancia de los HUFA en la salud humana

Los ácidos linoléico (LA, 18:2ñ-6) y α-linolénico (ALA, 18:3ñ-3) son considerados esenciales (EFA) para la mayoría de los vertebrados terrestres, ya que no pueden sintetizarlos *de novo*. Sin embargo su deficiencia en las dietas de los países industrializados no es común, ya que estos ácidos grasos están presentes en muchas de las plantas superiores que forman parte de nuestra

dieta. Los ácidos LA y ALA son precursores de los importantes HUFA, pero su síntesis a través de las series de elongasas y desaturasas es insuficiente en ciertos estadios de vida (Burdge and Wootton, 2002; Emken *et al.*, 1994; Gerster, 1998; James *et al.*, 2003; Nakamura and Nara, 2003). La síntesis de HUFA depende de la edad, el sexo, la ingesta, la presencia de enfermedades y otros procesos críticos del metabolismo. Por ello, a pesar de que LA y ALA son esenciales, su presencia no soluciona completamente la deficiencia de HUFA que puede reflejarse a largo plazo en una amplia variedad de problemas de salud (Bistrian, 2003). Dado que HUFA sí pueden revertir los síntomas de deficiencia LA y ALA por retro-conversión a ácidos grasos de cadena menor (Hansen *et al.*, 1986; Sprecher *et al.*, 1995), el DHA, EPA y ARA son también considerados esenciales para el mantenimiento saludable de las funciones vitales (Cunnane, 2000).

La ingesta de n-3 PUFA está asociada a una amplia variedad de beneficios para la salud (Simopoulos, 1999). Existen evidencias acerca de la mejora que producen los n-3 PUFA de la dieta en la prevención de enfermedades cardiovasculares (Lichtenstein, 2003), aterosclerosis (Ristic and Ristic, 2003), diversos cánceres asociados a hormonas (Terry *et al.*, 2003), diabetes (Brenner, 2003) y enfermedades auto-inmunes como la artritis reumatoide (Cleland *et al.*, 2003; Simopoulos, 2002). En concreto, el DHA esta implicado en la protección del tejido neuronal del estrés oxidativo (Yavin *et al.*, 2002), en el desarrollo de las neuronas (Wainwright, 2002) y la retina (Jeffrey *et al.*, 2001), la prevención de la enfermedad bipolar (Rapoport and Bosetti, 2002), hiperactividad (Burgess *et al.*, 2000), esquizofrenia (Kemperman *et al.*, 2006), depresión (Sontrop and Campbell, 2006) y Alzheimer (Cole *et al.*, 2005).

Aunque los niveles de HUFA en dieta dependen de los hábitos de los diferentes grupos sociales y países, la ingesta mínima recomendada es de al menos dos comidas basadas en pescado azul por semana y adulto. Así mismo se recomienda, independientemente de la cantidad de LA o ALA, la inclusión de DHA y ARA en las formulas infantiles (FAO/WHO, 1994). La dieta en los países occidentales es más rica en n-6 LA que en n-3 ALA ya que consumimos más aceites vegetales (n-6) que verduras (n-3) (Kris-Etherton *et al.*, 2000; Nettleton, 1991; Sanders, 2000). En ausencia de HUFA en la dieta, el desequilibrio corporal en el ratio n-3/n-6 se conserva, o aumenta mediante la desaturación y elongación hacia los HUFA. Dado que ambas familias de ácidos grasos son precursores de eicosanoides, su proporción (n-3/n-6) en dieta debería equilibrarse para asegurar una respuesta adecuada del metabolismo. Teniendo en cuenta que la ingesta de n-6 es alta en las sociedades occidentales, el ratio n-3/n-6 de la dieta debería ser equilibrado mediante la ingesta de DHA y EPA de 0.5 $g\ d^{-1}$ en niños, 1 $g\ d^{-1}$ en adultos y 2–4 $g\ d^{-1}$

en pacientes con hiperglicemia o con alto riesgo de enfermedades cardiovasculares (Kris-Etherton *et al.*, 2002). De momento, el pescado, incluyendo el producido por acuicultura, constituye la principal fuente de n-3 HUFA en la dieta.

El aceite de pescado como fuente de HUFA

Hoy en día, los aceites vegetales, ricos en ácidos grasos n-6, copan la mayoría del mercado mundial de aceites edibles (O'Mara, 1998). Los lípidos de origen animal como la manteca o la mantequilla son más baratos pero no son considerados tan saludables como los vegetales, ya que tienen muchas grasas saturadas y muy pocos PUFA. El aceite de pescado es la principal fuente de n-3 HUFA, en particular EPA y DHA (Nettleton, 1991). La harina y el aceite de pescado, la fuente tradicional de HUFA para la alimentación animal, se obtienen a partir de peces pequeños y grasos como la anchoa, el capelín y la polaca austral, que son espinosas y menos adecuadas para el consumo humano.

El aceite de pescado presenta un perfil de ácidos grasos complejo y variable. Su calidad depende de la especie, la estación, el lugar de pesca y su procesado. Los ácidos grasos más importantes son el DHA y el EPA, que están en un ratio de aproximadamente 10: 20. El aceite de soja, debido a s bajo precio, es tal vez la principal alternativa al aceite de pescado en el mercado pero, al igual que otras fuentes vegetales, carece de HUFA. Por ello, a pesar de que el aceite de soja es un 40 % más barato que el aceite de pescado (2006) (Josupeit, 2006), no resulta tan adecuado desde un punto de vista nutricional.

Perú es el principal productor mundial de aceite de pescado seguido de Escandinavia y Chile. Desafortunadamente las pesquerías de anchoveta de la costa peruana están muy influenciadas por los eventos climáticos. Durante el fuerte fenómeno de "El Niño" en 1998, las capturas disminuyeron radicalmente y precio del aceite de pescado se incrementó hasta los 750 US \$ $toneladas^{-1}$ (Figura 1.3). En condiciones climáticas normales, la producción anual de aceite de pescado se ha mantenido constante (1.0–1.7 millones de toneladas) en las últimas dos décadas, pero el precio ha ido aumentando gradualmente debido al crecimiento de la demanda. Al inicio del 2006 el precio del aceite de pescado alcanzó los 750 US \$ $toneladas^{-1}$, el máximo histórico que se alcanzó en 1998 debido a "El Niño", ha sido alcanzado hoy debido al aumento de la demanda de esta material prima. A pesar de que la Organización Internacional del Aceite y la Harina de Pescado (IFFO) confía en que el suministro del aceite de pescado será suficiente (Shepherd *et al.*, 2005), las principales pesquerías están controladas por cuotas pesqueras y por lo tanto

los stock no tienen capacidad para aumentar las capturas en respuesta a un previsible aumento de la demanda.

La acuicultura y la ganadería de cerdos, aves y rumiantes son los principales sectores de demanda global de harina y aceite de pescado. En concreto, la acuicultura es en la actualidad el sector alimentario que mayor crecimiento está experimentando, y ya produce un 33 % del pescado total consumido por el hombre. Se prevee que la producción global de acuicultura subirá hasta una tasa anual del 4.5 % durante los años 2010–2030 (Brugère and Ridler, 2004). Se trata, pues, del principal sector responsable del aumento de la demanda del aceite y harina de pescado (Naylor *et al.*, 2000; Tacon, 2004). Hasta el momento, la respuesta a este aumento de demanda ha sido aumentar el uso del aceite de pescado en los piensos para peces y crustáceos en detrimento de su uso para ganadería y fabricación de margarina. Lógicamente el motor de esta reorganización de los usos se basa en la optimización del valor nutricional de los n-3 HUFA. En vista del aumento inminente de la demanda de este producto para acuicultura, independientemente de la reorganización de sus usos, se teme que en un futuro no tan lejano la demanda podría exceder su producción.

Uno de los principales retos científicos para con la acuicultura es dar una solución sostenible al crecimiento del sector (Naylor *et al.*, 2000). Existen diferentes enfoques para prevenir que el aporte insuficiente de aceite de pescado pueda llegar a limitar el desarrollo de la acuicultura (Sargent *et al.*, 1999). En primer lugar, tal y como mencionábamos antes, la reorganización del uso del aceite de pescado ya está en proceso. El uso en piensos para animales terrestres está siendo redirigido hacia la acuicultura de especies marinas y hacia todo tipo de dietas de inicio que tienen requerimientos de HUFA más críticos. En segundo lugar, la producción del aceite de pescado debería optimizar su procesado y evitar la oxidación de los HUFA, para mejorar así su productividad. En tercer lugar, la sustitución parcial en piensos del aceite de pescado por aceites vegetales permitiría aliviar la presión que existe sobre los HUFA. El grado de sustitución de la dieta que permite mantener los niveles de crecimiento, bienestar del pez y calidad final del producto para el consumidor se ha definido para varias especies de peces carnívoros (Izquierdo *et al.*, 2003, 2005; Bell *et al.*, 2005; Ganga *et al.*, 2005). Todas estas medidas contribuyen, a través de la mejora en la eficiencia de conversión de peces pelágicos a peces de acuicultura, a disminuir la demanda que existe sobre el aceite de pescado, mejorando la sostenibilidad de la producción acuícola. Con todo, el planteamiento directo y definitivo al problema se encuentra, más allá de las soluciones parciales planteadas, en la producción de fuentes alternativas de HUFA.

Aceites unicelulares como fuente de HUFA

Los aceites unicelulares (SCO) o microbianos se extraen de la biomasa de los microorganismos producidos en cultivos comerciales (Ratledge, 1992). Este concepto es relativamente reciente en la industria de la biotecnología, pero está ganando interés debido a su capacidad para producir HUFA a escala industrial. Aunque hoy día el grueso de los HUFA en grandes cantidades y bajo precio está en el aceite de pescado, existe un gran interés en el SCO como sustituto potencial, ya que éste posee numerosas ventajas frente la fuente tradicional. El aceite de pescado está constituido por una compleja amalgama de más de 40 ácidos grasos diferentes cuya proporción depende de la especie y la estación del año, mientras que el SCO puede producir a lo largo de todo el año fuentes más concentradas de un ácido graso determinado (Tabla 11.1). Cada uno de estos ácidos grasos puede ser purificado con relativa facilidad ya que no existen otros PUFA tan abundantes. Existen ciertos aceites de pescado, como el aceite de órbita de atún, que presentan concentraciones dominantes de un ácido graso en particular (DHA) pero son escasos y caros (Saito *et al.*, 1997). Además, los aceites de pescado presentan el riesgo de convertirse en vehículos de contaminantes marinos que, en un momento dado, se puedan llegar a acumular en los animales de acuicultura (Hites *et al.*, 2004). Al contrario, los microbios oleaginosos se cultivan en condiciones controladas (bioreactores), obteniéndose un producto orgánico de calidad alimentaria y libre de contaminantes. Por último, la implementación del aceite de pescado en alimentos funcionales está limitada por los problemas de estabilidad oxidativa que presenta, así como del característico olor a pescado, mientras que el SCO posee una mayor estabilidad oxidativa gracias a los antioxidantes naturales o al empaquetado celular de los HUFA (Sargent *et al.*, 1997) y carece del olor o el sabor desagradable a pescado.

Los SCO son producidos por organismos oleaginosos, que son aquellos capaces de producir lípidos en cantidades que justifiquen su consideración comercial (Tabla 11.1). Habitualmente este valor es considerado por definición superior al 20 % del peso seco celular (CDW) (Ratledge, 2002b). Este valor incluye necesariamente una porción de lípidos no esenciales o de reserva. Los lípidos de reserva son acumulados dentro de la célula en triacilglicéridos, formando gotas de lípidos fáciles de extraer en el SCO. Únicamente unos pocos microorganismos (levaduras, hongos y algas) son capaces de acumular lípidos entre 20–70 % CDW en función de la especie de microbio y las condiciones ambientales. El proceso de acumulación de lípidos ha sido descrito para los organismos heterotróficos, que hasta el momento representan las únicas realidades comerciales dentro de los SCO ricos en HUFA (Ratledge, 2002a).

Cuadro 11.1: Principales ácidos grasos y productividades (r_{FA}) obtenidas en diversos organismos oleaginosos considerados como potenciales productores.

Organismo	14:0	16:0	16:1	18:0	18:1	18:2	18:3 n-6	20:4 n-6	20:5 n-3	22:5 n-6	22:6 n-3	r_{FA} (mg FA l^{-1} h^{-1})
Schizochytrium SR21[a]	13	29	12	1	1	1	-	-	-	12	36	145[b]
Schizochytrium ATCC20888[c]	-	-	-	-	-	-	-	-	-	-	25–40	≃417
Thraustochytrium aureum[d]	3	8	-	-	16	2	-	3	-	-	52	3
Ulkenia sp.[a]	3	30	-	1	-	1	-	-	-	11	46	-
Crypthecodinium cohnii[a]	20	18	2	-	15	0.4	-	-	-	-	39	53[e]
Nitzchia laevis[f]	23	33	-	-	33	2	1	1	4	-	-	10.4
Mortierella alpina 1S4[a]	0.2	6	-	2	4	4	2	70	-	-	-	54[g]
Mucor circinelloides[h]	22	-	1	6	40	11	18	-	-	-	-	4.8[i]

[a] (Ratledge, 2004), [b](Yaguchi *et al.*, 1997), [c](Bailey *et al.*, 2003), [d](Singh *et al.*, 1996), [e](de Swaaf *et al.*, 2003), [f](Barclay *et al.*, 1994; Kyle and Gladue, 1993), [g](Higashiyama *et al.*, 1998), [h](Ratledge, 2002b), [i](Kennedy *et al.*, 1993)

La lipogénesis generalmente se asocia a la limitación de un nutriente en el medio de cultivo, en concreto la fuente de nitrógeno (Gill *et al.*, 1977; Granger *et al.*, 1993; Ratledge, 2002b). La acumulación de lípidos se puede inducir manipulando el ratio entre la concentración de la fuente carbono y la de nitrógeno. En cultivo tipo "batch", este proceso suele ser bifásico. La primera fase se caracteriza por el rápido crecimiento celular y le sigue una segunda fase de estrés en la cual el carbono residual se redirige a la producción y acumulación de lípidos. Los eventos a nivel bioquímico que preceden la acumulación de lípidos fueron estudiados en detalle y recientemente revisados por Ratledge (2002b, 2004). Tras el agotamiento del nitrógeno en el medio, el poco amonio almacenado dentro de la célula en el AMP es secuestrado por acción de la AMP deaminasa. A continuación, la isocitrato dehidrogenasa (ICDH), la cual es dependiente del AMP (únicamente en los organismos oleaginosos), disminuye su actividad y en consecuencia el citrato se acumula dentro de la mitocondria para luego liberarse al citosol (Figure 1.4). Desde un punto de vista bioquímico, la acumulación de lípidos necesita de la presencia de la ATP: citrato liasa (ACL) responsable de la disociación del citrato en el citosol que provee de acetil-coA, principal componente estructural para la síntesis de ácidos grasos (Ratledge, 2002b). Esta enzima es crucial en la mayoría de los organismos oleaginosos pero no es la única responsable del proceso de acumulación de lípidos. La enzima málica es también esencial para este proceso, puesto que convierte el malato a piruvato, produciendo

reservas de NADPH que la célula necesita para la síntesis de lípidos. Cada extensión a la cadena alifática del ácido graso requiere de un acetil co-A y la reducción de dos moléculas de NADPH. Por ello, tanto la ATP: citrato liasa como la encima málica son cruciales para el aporte de los componentes necesarios para la síntesis de lípidos.

Microorganismos productores de DHA

Los microorganismos marinos, bacterias, algas y hongos, son los principales productores de HUFA en la cadena alimentaria del mar. Teniendo en cuenta la amplísima biodiversidad microbiana de los océanos (Sogin *et al.*, 2006) y el amplio porcentaje de especies que aún no han sido descritas (Colwell, 1997), los microorganismos marinos constituyen una buena fuente para la identificación y selección de productores potenciales de SCO. El DHA es un producto de alto valor añadido que tiene un buen potencial en biotecnología dado que no se puede producir de forma más barata por plantas terrestres. Las bacterias, aparentemente, no son adecuadas para la producción del DHA; no son capaces de acumular altas cantidades de triacilglicéridos y la presencia de ácidos grasos poco frecuentes, ausentes en los alimentos y piensos, puede dificultar su posterior utilización (Ratledge, 2002a). Los hongos del género *Mortierella* se cultivan para la obtención de ARA, sin embargo, las técnicas de cultivo de hongos no parecen ser las más apropiadas para la producción del DHA, debido a los largos periodos de fermentación de los organismos seleccionados y las bajas productividades que de ellos se derivan (Barclay *et al.*, 1994). Las algas marinas, ampliamente usadas en acuicultura como fuetes de HUFA, son capaces de mejorar radicalmente su productividad cuando son cultivadas en fermentadores (cutivo heterotrófico) en lugar de en cultivo clásico fototrófico (Apt and Behrens, 1999; Zaslavskaia *et al.*, 2001). El apantallamiento luminoso de las células en crecimiento fototrófico limita la máxima la penetración luminosa y la carga celular que se puede alcanzar en estos sistemas. Con el aumento del ratio superficie-volumen del medio de cultivo fototrófico se alcanzan ligeras mejoras en la densidades finales, pero también complica el escalado de las instalaciones. En consecuencia, el costo final de producción de biomasa en cultivo fototrófico al aire libre (US $ 20 kg^{-1}) o en fotobioreactores ($>$ US $ 50 kg^{-1}) (Borowitzka, 1997), no es competitivo con la biomasa cultivada en fermentadores de forma heterotrófica ($<$ US $ 5 kg^{-1}) (Gladue and Maxey, 1994).

Los microorganismos heterotróficos obtienen la energía necesaria para el metabolismo de la oxidación de un sustrato orgánico. Se cultivan en fermentadores en condiciones estériles, en oscuridad si es necesario, bajo condiciones ambientales muy controladas (temperatura, O_2 disuelto, aireación, agitación,

control del pH...). La estabilidad alcanzada en las condiciones de cultivo permite obtener, en una manera reproducible, un crecimiento rápido y una buena acumulación de lípidos en el organismo. Bajo condiciones óptimas de cultivo, con una adecuada aireación, ciertos organismos pueden producir hasta 100 g CDW l^{-1} en fermentadores de escala industrial. Estas altas densidades facilitan su implementación en un menor espacio y disminuyen el volumen de cosechado, que en otras circunstancias puede suponer hasta un 33 % del coste total de producción (Barclay *et al.*, 1987). Los principales factores que determinan el coste de producción del DHA son la escala de cultivo y la productividad del sistema (mg DHA l^{-1} h^{-1}) (Sijtsma *et al.*, 1998). A su vez la productividad del sistema depende de la tasa de crecimiento del organismo, de su contenido en lípidos y la proporción del DHA. El medio o las condiciones ambientales de cultivo permiten controlar el contenido en lípidos y el perfil de sus ácidos grasos. Por ejemplo, la utilización de sustratos de C_2, como el ácido acético o el etanol, parece mejorar la formación de lípidos al ofrecer a la célula precursores más directos del acetil co-A (Sijtsma and de Swaaf, 2004). El O_2 disuelto del cultivo permite controlar el grado de insaturación de los ácidos grasos de ciertos organismos que los sintetizan por medio de la acción de las enzimas desaturasas, dependientes del O_2. La aireación y la velocidad agitación del cultivo permite controlar el flujo O_2 al medio y así modelar en *Mucor circinelloides* la producción de unos lípidos con un perfil que imite el del aceite de cacao (Roux *et al.*, 1995). Actualmente, existen tres procesos comerciales para su producción del DHA que utilizan organismos pertenecientes a los thraustochytridos y dinoflagelados (Ratledge, 2004).

Los thraustochytridos son organismos marinos, heterótrofos obligados, capaces de desarrollar redes ectoplásmicas muy parecidas a las de los hongos. Inicialmente fueron clasificados dentro de los ficomicetos (hongos emparentados con las algas) por sus afinidades con los hongos, pero más adelante se alinearon dentro del reino cromista (Cavalier-Smith *et al.*, 1994). Sus características moleculares se encuentran más cercanas a las algas y forman, junto con los labyrinthulidos, un filum independiente dentro de las heterocontofitas. *Schizochytrium* sp. es un thraustochytrido cuyo contenido en ácidos grasos puede llegar hasta un 78.6 % de su CDW, entre los cuales el DHA representa un 33.3 % (Yaguchi *et al.*, 1997). La productividad final de DHA puede alcanzar los 150 mg l^{-1} h^{-1}, mientras que otros procesos basados en organismos emparentados con *Schizochytrium* sostienen unas productividades tan altas como 417 mg l^{-1} h^{-1} (Bailey *et al.*, 2003). Además del DHA, *Schizochytrium* sp. produce alrededor de un 10 % de ácido docosapentaen'oico (22:5 n-6; DPA-6) así como cantidades variables de ácidos grasos de cadena impar como el 15:0 y el 17:0. OmegaTech Inc., Boulder, CO, ahora propiedad

de Martek Biosciences Corp., produce comercialmente este organismo, pero los detalles del proceso no están disponibles al público. *Ulkenia* sp., otro thraustochytrido, tiene un perfil de lípidos muy parecido al de *Schizochytrium* sp. y se produce comercialmente en Nutrinova GmbH, Frankfurt, Alemania. Desafortunadamente, existe muy poca información disponible sobre este organismo.

Crypthecodinium cohnii, inicialmente conocido como *Gyrodinium cohnii*, fue aislado a partir de muestras de algas en descomposición. Este organismo marino pertenece al orden Dinophyceae y carece de cloroplastos. *C. Conhii* puede almacenar hasta un 50 % de su peso seco en lípidos que contienen DHA aproximadamente en un 32 % del total de ácidos grasos (TFA) (de Swaaf *et al.*, 2003). La máxima productividad de DHA referida hasta el momento para este organismo es de 53 $mg\ l^{-1}\ h^{-1}$. El DHA es prácticamente el único PUFA presente en este organismo, ya que otros PUFA suman menos del 1 % de TFA. Los exámenes clínicos del SCO de *C. cohnii* recibieron el estatus GRAS (Generally Recognized As Safe) que permite su consumo directo en humanos, mientras que *Schizochytrium* sp., con un perfil de lípidos más complejo, tan sólo posee el estatus GRAS para consumo animal (Ward and Singh, 2005). Martek Biosciences Corp. tiene la exclusividad para la producción de *C. cohnii* y su implementación como aditivos principalmente en fórmulas infantiles.

Los aceites microbianos están ganando interés debido a su implementación como metabolito de uso farmacéutico y principalmente como aditivo nutracéutico. Los SCO se añaden como alimentos funcionales en productos de consumo diario como la leche (Franklin *et al.*, 1999) o los huevos (Lewis *et al.*, 2000). En 2004, Nutrinova y Martek Biosciences absorbieron 19 % del mercado de omega-3 nutracéutico principalmente debido la implementación de aceites microbianos en las fórmulas infantiles (FPD, 2006). En acuicultura, las fuentes microbianas se usan principalmente en cultivo larvario, para cubrir la gran demanda de este nutriente que existe durante las etapas tempranas de crecimiento. Al menos dos compañías comercializan productos a partir de estos microbios para el enriquecimiento de las presas vivas, concretamente Aquafauna Bio-Marine Inc. y Advanced BioNutrition Inc.

Empleo de HUFA microbiano en acuicultura

Las fuentes HUFA derivadas de heterótrofos unicelulares se pueden combinar fácilmente para obtener el perfil de ácidos grasos óptimo para el enriquecimiento de presas vivas o para su implementación directa en dietas. Hasta el momento, la mayoría de estos organismos han sido empleados en la alimentación larvaria y sólo a nivel experimental como sustituto del aceite

de pescado en el grueso de los piensos de engorde para juveniles y adultos. Las dietas larvarias necesitan de un contenido en HUFA mayor y sus requerimientos nutricionales específicos justifican el uso de una fuente más cara pero también mejor controlada de HUFA. El ratio DHA: EPA en la mayoría de los aceites de pescado es alrededor de 10: 20 mientras que la larva necesita que las concentraciones de DHA sean dominantes en su dieta. A lo largo del enriquecimiento el ratio DHA: EPA sigue disminuyendo: Los protocolos de enriquecimiento se hacen en condiciones de alta aireación, durante 12–24 *h* en las cuales las emulsiones de enriquecimiento son susceptibles a la peroxidación (Mc Evoy *et al.*, 1995). Por otro lado la *Artemia*, que contiene algo de EPA pero nada en absoluto de DHA (Navarro *et al.*, 1993), es capaz de retroconvertir el DHA asimilado a EPA (Izquierdo, 1988; Evjemo *et al.*, 2001). Por lo tanto, es necesario comenzar con un alto ratio inicial de DHA: EPA del enriquecedor para mantener su calidad nutricional a lo largo del proceso de enriquecimiento y el consiguiente ayuno o "lavado" de las presas. Ya que este ratio es habitualmente muy inferior en el aceite de pescado, los microheterótrofos de origen marino, *Schizochytrium* sp. y *C. Cohnii*, presentarían la ventaja al tener un alto contenido de DHA. Su biomasa puede conservarse en preparados secos unicelulares y administrarse directamente a la presa. Este producto ofrece una fuente de lípidos encapsulada y por tanto protegida de la oxidación, que puede contener antioxidantes naturales, lípidos polares, proteínas, así como micronutrientes. Dado que el EPA se encuentra por debajo del 2 % TFA, estos organismos pueden proporcionar y mantener el ratio DHA: EPA adecuado a lo largo del proceso de enriquecimiento. Además, el hongo filamentoso *Mortierella spp.* es una buena fuente de ARA si este fuese necesario.

La biomasa de estos microheterótrofos fue implementada con éxito como enriquecimiento de presas vivas para el bacalao atlántico (*Gadus morhua*) (Cutts *et al.*, 2006; Park *et al.*, 2006), el eglefino (*Melanogrammus aeglefinus*) (Blair *et al.*, 2003), la cobia (*Rachycentron canadum*) (Faulk and Holt, 2005), el striped trumpeter (*Latris lineata*) (Battaglene *et al.*, 2006), la platija amarilla (*Limanda ferruginea*) (Copeman *et al.*, 2002), el fletán atlántico (*Hippoglossus hippoglossus*) (Shields and Irwin, 1998), la lubina estriada (*Morone saxatilis*), la lubina europea (*Dicentrarchus labrax*) y la dorada (*Sparus aurata*) (Harel *et al.*, 2002; Koven *et al.*, 2001). El enriquecimiento basado en *Schizochytrium* sp. o *C. cohnii* de la *Artemia* (Ritar et al., 2004) y de los rotíferos (*Brachionus plicatilis*) (Park *et al.*, 2006) aumentó el contenido de DHA alcanzando un ratio final DHA: EPA adecuado a los requerimientos larvarios. Las presas vivas, en particular la *Artemia*, retroconvierte el DHA a EPA, por lo tanto, *a priori*, podría no ser necesaria la implementación de otros enriquecedores microbianos de EPA. Las larvas de pescado obtienen

en general mejor crecimiento con los enriquecimientos basados en fuentes de DHA microbiana debido al alto ratio DHA: EPA que alcanzan las presas vivas en comparación con otros enriquecedores comerciales (Battaglene *et al.*, 2006; Cutts *et al.*, 2006; Harel *et al.*, 2002). Además, el enriquecimiento con productos basados en *Schizochytrium*, ofrecen DPA-6, el cual también es parcialmente retroconvertido a ARA por los rotíferos y la *Artemia* (Barclay and Zeller, 1996). Esto se refleja generalmente en el aumento del contenido de ARA y la consiguiente mejora en la supervivencia de la larva (Koven *et al.*, 2001; Park *et al.*, 2006). Algamac® (producto comercial hecho a base de *Schizochytrium*), incrementó la mortalidad de la *Artemia* debido a una mayor proliferción bacteriana durante el enriquecimiento, pero ello no afectó al cultivo de las larvas (Ritar *et al.*, 2004).

La inclusión de SCO directamente en las dietas microparticuladas debería reducir la pérdida de material asociada al proceso de enriquecimiento de presas vivas. A pesar de que los criaderos industriales aún dependen de las presas vivas para su producción, el desarrollo y la implementación de unas microdietas adecuadas podría ofrecer una alimentación con un mejor control nutricional, que en definitiva permita obtener una producción más predecible de alevines. La implementación de Algamac® en microdietas de inicio para dorada mejoró la incorporación de DHA en la larva (Robin and Peron, 2004). Además, la sustitución del aceite de pescado en la dieta para alevines de dorada por la biomasa *C. cohnii* en un 2 o 4 % mejoró el crecimiento y la supervivencia (Atalah *et al.*, in press). Por último la inclusión de *Thraustochytrid* ACEM 6063, un organismo emparentado con *Schizochytrium*, en dietas hechas con aceite de colza para juveniles de salmón atlántico, no afectó a la aceptación, el crecimiento o la función inmune de los peces pero incremento la mortalidad acumulativa tras el trasvase al agua salada o tras el reto al patógeno *Vibrio anguillarum* (Carter *et al.*, 2003). Sin embargo, cuando la sustitución del aceite de pescado en las dietas de parr de salmón fue más completa no se observó ningún efecto nocivo (Miller *et al.*, in press). El subproducto de la biomasa de *Mortierella alpina* obtenido tras la extracción de su aceite, fue incluido en dietas para los reproductores de lubina estriada (*Morone saxatilis*) incrementando la tasa de eclosión de sus huevos (Harel *et al.*, 2002).

La dorada como modelo para el estudio de la implementación de organismos heterótrofos como fuente de HUFA

La dorada (*Sparus aurata*) es un teleósteo marino (familia *Sparidae*) que ha sido tradicionalmente cultivado de modo extensivo en los esteros de la cuenca mediterránea (Ej. Cádiz). Sin embargo, no ha sido hasta los años ochenta que este pescado de consumo ha empezado a cultivarse de forma intensiva. Desde entonces, la producción mundial de esta especie aumentó hasta las 91 100 toneladas anuales en 2005 según la Federación Europea de (FEAP). Hoy en día, la producción piscícola de dorada de acuicultura es la tercera en volumen tras la producción de salmón y trucha. Se trata de una industria consolidada que produce el 92 % de toda la dorada vendida en lonja, mientras que el otro 8 % corresponde a la dorada de origen extractivo (2003). Los principales países productores en el 2005 fueron Grecia (55 %), España (17 %), Turquía (17 %) e Italia (9 %) (Apromar, 2006).

Las técnicas de cultivo de la dorada están bien desarrolladas y sus requerimientos nutricionales se usan como modelo para confeccionar piensos para especies menos desarrolladas como la lubina europea (*Dicentrarchus labrax*) o el besugo (*Pagrus pagrus*). En particular, el metabolismo de los lípidos está bien estudiado y sus requerimientos detallados para cada uno de los estadios de crecimiento. La nutrición larvaria es más delicada y necesita un perfil de lípidos muy determinado. Por estos motivos, la dorada se considera una especie ideal para evaluar el efecto de la inclusión fuentes microbianas en los piensos en los distintos aspectos de la nutrición de peces.

11.2. Objetivos

A la vista de la importancia de los HUFA, en particular del DHA, para los peces marinos y de la dependencia que sufre la acuicultura por el suministro incierto del aceite de pescado como fuente de esos ácidos grasos, esta tesis pretende estudiar la viabilidad de los microorganismos heterótrofos como alternativa que permita un desarrollo sostenible de la acuicultura. Con este objetivo y debido a las ventajas anteriormente referidas, se seleccionaron dos organismos heterótrofos, *Schizochytrium* sp. y *Crypthecodinium cohnii*, como fuentes alternativas de DHA para las larvas de dorada *Sparus aurata*. Con la intención de desarrollar un proceso integrado se definieron varios objetivos específicos:

1. Estudiar la fisiología y la bioquímica de la acumulación de lípidos en *Schizochytrium* sp. y *C. cohnii* utilizando distintos sistemas de cultivo,

variando la composición del medio y las condiciones de aireación.

2. Optimizar el sistema de fermentación de ambos organismos mediante la implementación de los parámetros y técnicas de cultivo que modulen la bioquímica de los lípidos y en última instancia la acumulación de DHA.

3. Determinar el efecto de la sustitución del aceite de pescado por la biomasa de *Schizochytrium* sp. y *C. cohnii* en microdietas para dorada, examinando la ingesta de la dieta y el crecimiento y supervivencia larvaria.

4. Comprobar el efecto de ambas algas en la salud larvaria, tanto en la respuesta al estrés como en la resistencia a enfermedades.

5. Analizar el efecto que tienen ambas algas en el metabolismo de los lípidos mediante el estudio de la transformación del perfil de lípidos de las larvas.

11.3. Materiales y métodos generales (2)

La parte experimental de la tesis fue estructurada en dos grandes bloques enfocados a la fermentación microbiana y a la nutrición larvaria de peces. El trabajo de fermentación se realizó en el Lipid Research Centre, del Departamento de Ciencias Biológicas de la Universidad de Hull (Inglaterra). Los experimentos sobre nutrición de las larvas de dorada (*Sparus aurata*) se desarrollaron en las instalaciones de Cultivos Marinos del Grupo de Investigación en Acuiculture (ULPGC & ICCM) (España). Los detalles de la metodología empleada en cada experimento se especifican en el apartado de material y métodos de cada capítulo.

11.4. Discusión

Discusión 3: La acumulación de lípidos producidos por *Schizochytrium* G13/2S en cultivo continuo.

La acumulación de lípidos en los microorganismos oleaginosos se induce mediante la limitación de un nutriente distinto de la fuente de carbono, habitualmente el nitrógeno, (Gill *et al.*, 1977; Ratledge and Wynn, 2002). Durante el cultivo tipo "batch", el crecimiento en biomasa (inicialmente pobre en lípidos) cesa, y los lípidos comienzan a acumularse a lo largo de la siguiente fase lipogénica. Sin embargo, según se observa en el presente experimento, durante el cultivó tipo "batch", en presencia de glucosa, *Schizochytrium* G13/2S

sintetizó lípidos de reserva durante la fase de crecimiento, indicando que este organismo presenta un patrón de acumulación de lípidos diferente del habitualmente observado en los organismos oleaginosos (Ratledge, 2002a). Este patrón particular también se observó en *Cryptococcus terricolus* (Boulton and Ratledge, 1984) y *Mortierella alpina* LPM301 (Eroshin *et al.*, 2002). Durante el cultivo en continuo *Schizochytrium* G13/2S fue capaz de asimilar y también acumular fuentes de nitrógeno del medio, como el glutamato. Esta capacidad de modificar su composición interna y almacenar nitrógeno intracelular permitiría a la célula seguir creciendo después de que el nutriente limitante haya sido absorbido del medio, justificando así la simultaneidad entre el crecimiento y la acumulación de lípidos hallada en ésta célula. A pesar del peculiar patrón de formación de lípidos, el nitrógeno también indujo la acumulación de lípidos en *Schizochytrium*, confirmando la importancia de este parámetro. El cultivo continuo permitió identificar el nitrógeno como nutriente limitante, aislando su efecto respecto al de otras variables, incluida la tasa de crecimiento. El crecimiento de las células fue proporcional a la concentración de la fuente de nitrógeno en el medio de bombeo y una concentración de 2 g glutamato l^{-1} limitó la carga del sistema. El aumento del contenido de ácidos grasos, al reducir la concentración de glutamato, con una proporción constante de glucosa, sugiere que la limitación de la fuente de nitrógeno es un parámetro determinante para la acumulación de lípidos en *Schizochytrium* G13/2S. Por lo tanto la producción de DHA puede ser modulada mediante el control de la fuente de nitrógeno.

En ausencia de una fuente de carbono, los microorganismos oleaginosos usan sus lípidos de reserva para el crecimiento y el mantenimiento celular. Por ejemplo, durante el cultivo continuo en condiciones limitantes de carbono, *Candida* 106 redujo su contenido máximo de lípidos hata el 14 % TFA (Gill *et al.*, 1977). Durante el cultivo tipo "batch" en ausencia de glucosa, *Schizochytrium* G13/2S fue capaz de usar sus lípidos de reserva y crecer hasta que su contenido de lípidos alcanzó tan solo el 10 % del CDW, lo cual indica que es el mínimo constitutivo necesario para crecer. Sin embargo, durante el cultivo continuo con déficit de glucosa su nivel de lípidos fue de de 22 %. Este porcentaje no responde únicamente a los lípidos estructurales ya que las gotas de aceite acumulado eran visibles a través del microscopio. Por lo tanto, la acumulación de lípidos durante el cultivo continuo limitado por la fuente de carbono es una peculiaridad de *Schizochytrium* que aún no había sido descrita en otros organismos oleaginosos.

El grado de instauración y el perfil de los ácidos grasos de los organismos oleaginosos producidos en cultivo continuo se puede modificar variando la concentración de las fuentes de carbono y nitrógeno (Gill *et al.*, 1977), el tipo de sustrato, la tasa de dilución (Evans and Ratledge, 1983), el O_2 disuel-

Cuadro 11.2: Comparación del crecimiento, la productividad de biomasa (r_{CDW}), productividad de DHA (r_{DHA}), la tasa específica de formación de DHA (μ_{DHA}), conversión a biomasa y la acumulación de ácidos grasos totales (TFA) en *Schizochytrium* G13/2S bajo dos sistemas de cultivo tipo "batch" y un cultivo tipo continuo.

Tipo de cultivo[a]	Batch		Continuo
Tiempo (h)	44	56	Estado de equilibrio
Peso seco celular (CDW) ($g\ l^{-1}$)	15	15.7	8
r_{CDW} ($g\ l^{-1}\ h^{-1}$)	0.33	0.27	0.32
r_{DHA} (mg DHA $l^{-1}\ h^{-1}$)	50	46	40
μ_{DHA} (mg DHA g CDW$^{-1}\ h^{-1}$)	3.4	2.9	4.9
Conversión (g CDW g^{-1} glucose)	0.39		0.39
TFA (% w/w of CDW)	35	35	30
Residuos de ácidos grasos (% w/w of TFA)			
14:0	7	5	4
16:0	33	32	37
18:0	2	2	3
22:5 n–6	12	10	10
22:6 n–3	43	47	41
Otros Ácidos grasos	3	4	5

[a] El medio de cultivo "batch" y continuo presentan la misma concentración de glucosa (40 $g\ l^{-1}$) y de ácido glutámico monosódico (2 $g\ l^{-1}$). El inóculo del cultivo tipo "batch" fue del 0 % v/v y la tasa de dilución en el cultivo continuo de 0.04 h^{-1}. Los análisis se realizaron de acuerdo a lo establecido en la Tabla 3.1.

to (Roux *et al.*, 1995) y la temperatura (Kendrick and Ratledge, 1992). Sin embargo, el perfil de lípidos en *Schizochytrium* G13/2S presentó una fuerte preponderancia por el ácido palmítico, el DPA y el DHA bajo todas las condiciones estudiadas (Tablas 3.1–3.2), lo cual indica que la manipulación fisiológica del perfil de ácidos grasos parece ser más difícil que en otros organismos. Aún así, el cultivo continuo muestra que las concentraciones de carbono y nitrógeno del medio pueden modificarse para inducir la acumulación de lípidos durante la fase de crecimiento de *Schizochytrium* G13/2S, permitiendo una acumulación óptima de DHA.

La producción de lípidos y otros metabolitos secundarios responde habitualmente a un patrón bifásico, por lo que el cultivo a nivel industrial es mayoritariamente de tipo "batch". Sin embargo, entre los organismos que son capaces de crecer y acumular lípidos simultáneamente, el cultivo continuo monofásico puede presentarse como una alternativa adecuada para la producción de lípidos y DHA. Entre el rango de diluciones aplicado, los 0.04 h^{-1} cosechó las mejores r_{DHA}. El análisis detallado entre el cultivo tipo "batch" y el cultivo continuo (D=0.04 h^{-1}) utilizando un medio idéntico revela el potencial de las distintas estrategias de cultivo (Table 11.2). A pesar de que la r_{DHA} del cultivo tipo "batch" (50-46 mg DHA l^{-1} h^{-1}) es aparentemente superior a la del cultivo continuo de *Schizochytrium* G13/2S r_{DHA} (39 mg DHA l^{-1} h^{-1}), recalculando este valor para el cultivo tipo "batch", teniendo en cuenta un tiempo de reinicio del ciclo de 24 h necesario para volver a preparar el fermentación, nos daría un valor final de 32 mg DHA l^{-1} h^{-1} en comparación con los 39 mg DHA l^{-1} h^{-1} alcanzados en el cultivo continuo. Además, el proceso de cosechado durante un cultivo continuo (Ej. centrifugación continua) es mucho más eficiente que el cosechado de un solo volumen obtenido tras el final del ciclo "batch". Dado que la conversión de la glucosa es similar para ambos tipos de cultivo, y que la cantidad de glucosa en cultivo continuo puede ajustarse a las necesidades del cultivo continuo, la mejora en la r_{DHA} podría suponer una ventaja económica en el proceso de fermentación, destacando así el potencial del cultivo continuo de *Schizochytrium*, a tasas de dilución óptimas, para la producción de DHA.

Discusión 4: El efecto de la aireación en la acumulación de lípidos de *Schizochytrium* G13/2S y *Crypthecodinium cohnii*.

Durante el cultivo de los microorganismos bajo limitación de O_2, disminuye el reciclaje de los metabolitos secundarios (ej. NADPH), que se realiza mediante la fosforilación oxidativa en la cadena de transporte de electrones (Tsai *et al.*, 1995). El paro metabólico resultante de la falta de O_2 podría ser responsable del bajo crecimiento observado en los experimentos de *Schizochytrium* G13/2S y *Crypthecodinium cohnii*. A su vez, el bajo crecimiento alcanzado permitiría que otros nutrientes, como el nitrógeno, se conserven sin utilizar. El nitrógeno que en condiciones limitantes induce la acumulación de lípidos (Ganuza and Izquierdo, 2007; Ratledge, 2004), en exceso inhibe su acumulación, de acuerdo con lo observado en los tratamientos con baja aireación. En estas condiciones, la célula utilizaría el poco NADPH disponible para la síntesis de proteínas dejando una cantidad limitada de esta

molécula implicada en la síntesis de lípidos (Ratledge, 2004). En consecuencia, podríamos observar que las bajas concentraciones de O_2 se relacionan con la mala acumulación de lípidos en ambas especies oleaginosas, presumiblemente debido a la acumulación de NADH y nitrógeno. El patrón de acumulación de lípidos en *Candida* 107 (TFA) (Hall and Ratledge, 1977) y *Phaffia rhodozyma* (astaxantina) bajo distintos niveles de O_2 fue muy similar al observado en el presente experimento (Yamane *et al.*, 1997).

La síntesis de ácidos grasos insaturados está tradicionalmente relacionada con la actividad de las desaturasas de ácidos grasos, que son unas enzimas dependientes del O_2 (Klein and Volkmann, 1975; Moreton, 1988). Esta condición se refleja en una amplia variedad de levaduras que, en condiciones de bajo O_2, disminuyen el índice de instauración de sus ácidos grasos (Roux *et al.*, 1995; Valero et al., 2001). En el presente experimento, la proporción de los ácidos grasos poliinsaturados se mantuvo constante bajo las diferentes condiciones de aireación, indicando que la actividad de desaturación no estuvo afectada por la limitación del O_2. A pesar de que estas enzimas tienen una gran afinidad por el O_2, incluso en condiciones limitantes de este nutriente (Meyer and Bolch, 1963; Klein and Volkmann, 1975), la baja aireación en *C. cohnii* disminuyó la síntesis del ácido oléico (OA; 18:1 n-9), indicando que la falta de O_2 inhibió la actividad de la Δ^9-desaturasa. De igual modo, el aumento de la transferencia del O_2 con n-dodecano durante la fermentación de *C. cohnii* CCMP316, disminuyó la relación entre el DHA y el OA (Lopes da Silva et al., 2006). Por lo tanto, la síntesis de DHA y OA en *C. cohnii* parece estar afectada de forma diferente por la aireación. Sonnenborn y Kunau (1982) observó que el complejo FAS (ácido graso sintetasa) aislado a partir del citosol de *C. cohnii* produjo tan solo ácidos grasos saturados, indicando que la desaturación del OA fue probablemente producto de una desaturasa asociada a la membrana. Por otro lado el uso de precursores radioactivos demostró que el OA no es un precursor de DHA ya que este HUFA se sintetiza *de novo* por una molécula de dos carbonos (C_2), y no a partir de otros ácidos grasos asimilados del medio (Beach et al., 1974; Henderson and Mackinlay, 1991). En consecuencia, la biosíntesis del OA está aislada de la del DHA lo cual ayuda a entender por qué ambos ácidos grasos tuvieron diferentes respuestas a la aireación durante el cultivo de *C. cohnii*.

En el presente experimento, el régimen de aireación no varió la proporción de DHA de ninguno de los organismos. Además, *Schizochytrium* G13/2S fue capaz de sintetizar tanto DHA como DPA en un fermentador en ausencia casi absoluta de O_2. Los resultados obtenidos durante la fermentación anaerobia están en concordancia con la nueva ruta de síntesis independiente al O_2 (poliketido sintasa; PKS) que aparentemente actúa con un menor gasto energético de NADPH en *Schizochytrium* (Metz et al., 2001). La síntesis del

DHA mediante el PKS de tipo anaeróbico también en *C. cohnii* explicaría por qué este alga no presenta PUFA de cadena intermedia al DHA y también por qué, cuando se prolonga la rampa de temperatura del GC, se observa el largísimo y nada común ácido octacosaoctaenoic (28:8n3), también presente en algunos thraustochytridos (Van Pelt *et al.*, 1999). De acuerdo con este planteamiento, se observó que las proporciones del DHA en los lípidos de *C. cohnii* no se vio afectada por la presencia de los inhibidores de la Δ^4, Δ^6 (de Swaaf et al., 2003) y Δ^{15} desaturasa (Henderson *et al.*, 1990). A estas evidencias en defensa de la síntesis por la ruta PKS tanto en *Schizochytrium* sp. como en *C. cohnii*, el presente estudio contribuye indicando que el DHA tampoco se vio afectado por la aireación. Desde el punto de vista del proceso de fermentación, los resultados demuestran que la proporción de DHA en los ácidos grasos no está directamente afectada por las bajas concentraciones de O_2 disuelto que frecuentemente caracteriza la fermentación a gran escala.

Discusión 5: La restricción en los precursores de ácido propiónico inhibe la síntesis de ácidos grasos de cadena impar en *Schizochytrium* G13/2S.

Los ácidos grasos de cadena impar (OCFA) proceden principalmente de los organismos procariotas. Los productos lácteos, debido a la actividad bacteriana del rumen de la vaca, contienen hasta un 2 % de OCFA (Chouinard *et al.*, 1998; Vlaeminck et al., 2006). Los thraustochytridos, entre los que se incluye *Schizochytrium* G13/2S, también son capaces de sintetizar OCFA (15:0 y 17:0) hasta un 40 % TFA (Wang *et al.*, 2000; Fan et al., 2001). Los OCFA se acumulan tanto en los lípidos neutros como en los polares, con especial preferencia por la fosfatidilcolina (Abe *et al.*, in press). Los efectos fisiológicos de la nutrición de estos ácidos grasos son desconocidos, sin embargo, en el presente estudio se consiguió disminuir los niveles de los OCFA en *Schizochytrium* hasta los alcanzados en los productos lácteos. Por ello, la producción de OCFA por este alga no debería comprometer su valor nutritivo. Por otro lado, la disminución en la producción del 15:0 y el 17:0 aumentó el contenido de DHA desde el 39 al 45 % TFA, que es el principal objetivo de la producción de este aceite microbiano. Por lo tanto, los resultados indican que, independientemente de sus implicaciones nutricionales, la producción tanto de 15:0 como de 17:0 puede disminuir el porcentaje del resto de ácidos grasos, incluyendo el valioso DHA.

La presencia de proteosa de peptona y extracto de levadura en el cultivo de *Thraustochytrium* sp. incrementó el % de los OCFA en relación al ratio levadura/peptona (Wang *et al.*, 2000). Sin embargo, *Schizochytrium sp.* cul-

tivado en un medio con extracto de levadura como única fuente de nitrógeno produjo un 40 % de OCFA (Fan et al., 2001). En el presente experimento, la proporción de los OCFA en los lípidos de *Schizochytrium* G13/2S aumentó con la concentración de las fuentes de nitrógeno hasta un 11 % TFA. Al disminuir concentración de extracto de levadura y peptona de proteosa disminuyó el % de OCFA en *Schizochytrium* sp. indicando que el propionato, o algunos precursores de este compuesto están presentes en dichos ingredientes y son utilizados como fuente de carbono. Esta molécula de tres carbonos (C_3) se incorpora a la maquinaria de síntesis de ácidos grasos en lugar de acetil co-A formando una cadena alifática impar (Ingram *et al.*, 1977). En consecuencia, se observó que la implementación de fuentes de nitrógeno controladas, carentes de precursores de ácido propiónico como el amonio o el glutamato, disminuyó el contenido de OCFA a un mínimo (2 % TFA), el cual parece ser inherente al metabolismo específico de este organismo. En resumen, el medio de cultivo desarrollado disminuyó en 5-veces la cantidad de los OCFA en beneficio del DHA el cual aumentó de 39 a 45 %, demostrando la plasticidad del perfil de lípidos de *Schizochytrium* G13/2S. Estos resultados subrayan la importancia de desarrollar medios completamente definidos que se ajusten a los requerimientos de cada microbio y proceso.

Discusión 6: Cultivo en altas densidades celulares de *Schizochytrium* G13/2S en un sistema "fed-batch" de amonio controlado en pH-auxostato.

Las especies del género *Schizochytrium* se producen comercialmente en cultivo tipo "batch" o "fed-batch", bajo condiciones limitantes de nitrógeno (Ratledge, 2002a). En estas condiciones se pueden alcanzar los 200 g peso seco l^{-1} (Bailey *et al.*, 2003), sin embargo, en el cultivo a gran escala, debido a las dificultades que presenta su bombeado y cosechado, las densidades suelen ser inferiores. Generalmente, la producción de aceites microbianos comienza con una serie de fermentadores cada vez de mayor tamaño, cuyo objetivo es el producir rápidamente el máxima concentración "práctica" de biomasa. A continuación, en una segunda etapa, toda esta biomasa se transfiere a un último fermentador, en un medio formulado de forma que el organismo alcanza condiciones limitantes de nitrógeno e incrementa su carga de lípidos. Para que la célula acumule lípidos, es necesario que la ausencia de nitrógeno inhiba la síntesis de nuevas proteínas y, en consecuencia, el flujo de carbono que proviene de la asimilación de la glucosa se dirija hacia la síntesis de ácidos grasos. Por ello, hasta este último fermentador, el medio de cultivo está diseñado de una forma equilibrada, permitiendo que el cultivo alcance el

máximo crecimiento celular con un contenido habitualmente bajo en lípidos. El presente estudio se encuadra precisamente en esta primera fase del cultivo. El sistema aquí implementado permite corregir automáticamente la concentración de amonio mediante el control del pH, manteniendo un suministro de nitrógeno equilibrado.

Los cultivos realizados tanto en matraces como en fermentadores demostraron que *Schizochytrium* G13/2S crece bien con glucosa y amonio. En concordancia con el trabajo de Yaguchi *et al.* (1997), el amonio (como sal de tartrato) no resultó tóxico a concentraciones de hasta 30 $g\ l^{-1}$. En concreto, se observó que el crecimiento es mayor a concentraciones menores de NH_4^+ (aprox. 1.7 $g\ l^{-1}$), indicando que el cultivo "fed-batch" de NH_4^+ puede ser útil para optimizar la tasa de crecimiento. De hecho, la productividad de biomasa del "auxostato" (1.3 g CDW $l^{-1}h^{-1}$) duplicó la productividad obtenida por Yaguchi y colaboradores en cultivo "fed-batch" (0.53 g CDW $l^{-1}h^{-1}$). Las altas concentraciones de glucosa toleradas por el organismo (alrededor de 150 gl^{-1}), serían insuficientes para alcanzar las densidades óptimas (60–80 gl^{-1}) y el contenido de lípidos máximo (40–45 %) para su producción comercial, por lo que se recomienda incluir más glucosa durante el cultivo.

A pesar de que el objetivo directo del presente estudio, que se realizó en condiciones suficientes de nitrógeno, no fue la acumulación de lípidos, las altas tasas de crecimiento obtenidas permitieron alcanzar productividades DHA dentro del rango descrito por Yaguchi *et al.* (1997) en condiciones limitantes de nitrógeno. El contenido de ácidos grasos en el presente experimento fue del 25 % CDW, muy por encima de lo esperado en condiciones no limitantes de nitrógeno. Por lo tanto, el cultivo alcanzó ya en ésta primera fase más de la mitad del contenido de lípidos presente en la biomasa comercial de *Schizochytrium* sp. (Barclay *et al.*, 2005). En consecuencia, una vez que el fermentador/auxostato haya alcanzado unos 50 g CDW l^{-1} con un 25 % de lípidos, un simple cambio de la solución alcalina que controla el pH (de NH_4OH a KOH) inducirá a la fase acumulación de lípidos durante la cual la célula podría aumentar su contenido hasta 45 %. Previsiblemente, durante esta fase, el aumento de la biomasa se debería principalmente al aumento del contenido en lípidos.

El presente cultivo de *Schizochytrium* utiliza un medio completamente definido, en el cual las sales marinas habitualmente utilizadas en el cultivo de estos organismos se sustituyeron por NaCl con el objeto de mantener la presión osmótica adecuada. Asimismo, se omitieron fuentes complejas de nitrógeno como el extracto de levadura o la proteosa de peptona, evitando así fuentes de ácido propiónico (ver Capítulo 5). De producirse este ácido e incorporarse en los ácidos grasos daría lugar a ácidos grasos de cadena impar, que son producidos por este organismo en cantidad moderada (Shirasaka *et*

al., 2005).

En conclusión, el sistema NH_4/pH-auxostat se presenta como una técnica prometedora para la primera fase de producción de *Schizochytrium* sp. debido a su capacidad para mantener un alto crecimiento y producir simultáneamente una cantidad sustancial de lípidos y DHA.

Discusión 7: Producción de ácido docosahexaenóico por *Crypthecodinium cohnii* cultivado en un sistema "fed-batch" con etanol.

El etanol es una fuente de carbono prometedora para la producción de DHA en *C. cohnii*. El factor de conversión a biomasa es de unas 2.4 veces mayor que para el ácido acético (de Swaaf et al., 2003b). Además, es relativamente barato y menos corrosivo que el ácido acético. Se trata de una molécula de dos carbonos C_2, precursora directa de la pieza básica para la síntesis de ácidos grasos, el acetil co-A, y por lo tanto se consideran un sustrato ideal para la producción de lípidos (Sijtsma and de Swaaf, 2004). En este experimento, el contenido de ácidos grasos fue ligeramente menor del esperado para sustratos C_2 (de Swaaf et al., 2003b), por ello las buenas productividades alcanzadas en esta fermentación de etanol fueron principalmente debido al rápido crecimiento de la cepa empleada y no al aumento al incremento en el contenido en lípidos o DHA de la célula.

Se cree que *C. cohnii* tiene una alta demanda por el O_2, el cual contribuye, en condiciones limitantes, a disminuir su crecimiento y su contenido en lípidos (en el Capítulo 4). En cultivos muy poblados, el O_2 disuelto puede resultar factor limitante. Lógicamente el escalado a fermentadores industriales aumentará este problema ya que volúmenes mayores ofrecen un menor coeficiente de transferencia de O_2 (KLa) (Cheng, 1998). En este experimento se usó un fermentador de 10 l con la intención de producir suficiente biomasa para el trabajo experimental en acuicultura. Durante gran parte de la fermentación el O_2 disuelto se mantuvo por debajo del 5 %, sugiriendo que el O_2 podría estar limitando el crecimiento, la producción de lípidos y de DHA. Sin embargo, cuando la fermentación se repitió manteniendo el O_2 disuelto por encima del 30 % de saturación, no se observó ninguna de las mejoras esperadas en el crecimiento o la acumulación de lípidos. El etanol se consumió a la misma tasa de alimentación (2 ml etanol l^{-1} h^{-1}) que en la fermentación que no se enriqueció con O_2. Estos resultados sugieren que debe haber algún otro factor limitante en el cultivo.

Se compararon los principales parámetros de la presente fermentación con el trabajo hecho por de Swaaf *et al.* (2003) (Tabla 11.3). En su trabajo de

Cuadro 11.3: Comparación entre el cultivo tipo "fed-batch" de *Crypthecodinium cohnii* utilizando ácido acético o etanol.

	Sustrato añadido		
Cepa (*C. cohnii*)	Ácido acético ATCC 30772[a]	Etanol ATCC 30772[a]	Etanol ATCC50060
Tiempo de fermentación (h)	210	200	210
Capacidad del fermentador (l)	2	2	10
Peso seco celular (CDW) ($g\ l^{-1}$)	59 ±3	77 ±3	91
cidos fgrasos totales (TFA) (%)	50 ±1	41 ±1	34
DHA (% TFA)	32 ±0	33 ±0	39
Concentración de TFA ($g\ l^{-1}$)	30 ±1	31 ±1	34
Concentración de DHA ($g\ l^{-1}$)	9.5 ±0.1	10.1 ±0.4	12
Conversión a biomasa (g (g substrate)$^{-1}$)	0.13	0.31	0.31
Productividad de DHA (r_{DHA})	44.5 ±0.5	51.4 ±2.1	57
Máxima tasa de alimentación ($ml\ l^{-1}\ h^{-1}$)	pH-auxostat	3	2

[a] Los datos sobre la cepa ATCC 30772 se obtubieron de de Swaaf *et al.* (2003); de Swaaf et al. (2003b) y representa la media de dos fermentaciones. Los distintos parámetros fueron calculados para el punto que representa el declive o el final de la fermentación.

Swaaf utilizó un fermentador menor, de 2 l de capacidad, con una agitación de hasta 1250 rpm y una aireación de 1 $l\ min^{-1}$. Además, se añadió al medio 0.5 $g\ l^{-1}$ de una solución enzimática (Glucanex, Novo Nordisk, Neumatt, Suiza) con la intención de reducir la viscosidad del medio y mejorar así la transferencia del O_2. Las condiciones de oxigenación del cultivo, incluyendo el reducido tamaño del fermentador que se empleo en la experiencia primera, pudieron contribuir a mantener el O_2 disuelto por encima del 30 % de saturación. En nuestro experimento, el O_2 disuelto se mantuvo por encima del 30 % mediante el enriquecimiento del aire con O_2. El enriquecimiento no presentó mejoras en el crecimiento o el contenido en lípidos o DHA respecto a la fermentación sin enriquecimiento de O_2, sugiriendo que el O_2 no era un factor limitante. En consecuencia la mejora en la r_{DHA} del presente experimento parece estar protagonizada por la cepa empleada, la ATCC 50060 y no por a falta de O_2.

Estos resultados vienen a confirmar el potencial del método "fed-batch" de etanol para la producción de DHA por *C. cohnii*, mostrando como la cepa ATCC 50060 alcanza una biomas record de 91 $g\ l^{-1}$ en 210 h de fermentación. Por ello, la r_{DHA} final (56 mg DHA $l^{-1}\ h^{-1}$; utilizando un 10 % inóculo) fue mayor que la anteriormente descrita para este método. Los próximos esfuerzos de optimizacion de este sistema de cultivo deberían concentrarse en otros parámetros que el O_2 disuelto ya que este no fue crítico en las condiciones investigadas.

Discusión 8: La implementación como sustitutos del aceite de pescado de *Crypthecodinium cohnii* y *Schizochytrium* sp. en las microdietas para dorada (*Sparus aurata*).

Se seleccionaron dos cepas de distintas microalgas heterótrofas por su capacidad de producir DHA, un ácido graso esencial que habitualmente se obtiene del aceite de pescado. Tanto *Schizochytrium* G13/2S como *Crypthecodinium cohnii* ATCC 50060, fueron previamente analizados y sus respectivos cultivos optimizados mediante la implementación de diferentes medios y tecnologías de fermentación (de Swaaf *et al.*, 2003; Ganuza *et al.*, in press). Finalmente, la fermentación de *Schizochytrium* sp. resulto ser más estable y productiva que la de *C. cohnii*. La biomasa producida por ambas microalgas presentó un alto contenido de DHA y un bajo contenido en carbohidratos y cenizas. Estas características facilitan la manipulación del perfil de ácidos grasos de la dieta mediante la implementación con un grado de inclusión moderado de las microalgas. Teniendo en cuenta que la mayoría de los aceites de pescado presentan mayores niveles de EPA que de DHA, el enriquecimiento con DHA puede resultar muy interesante para ciertas aplicaciones en acuicultura con mayores requerimientos por este ácido graso. Por ejemplo, el cultivo larvario de peces, posee requerimientos específicos hacia el DHA mayores que los de cualquier otro estadio (Izquierdo, 1996), si bien este ácido graso es difícil de concentrar a partir del aceite de pescado debido a la presencia de otros PUFA durante la purificación.

La utilización de estas microalgas en las microdietas para la dorada (*S. aurata*) permitió que las larvas alcanzasen el mismo nivel de DHA que aquellas alimentadas con dietas hechas con aceite de pescado, demostrando que son adecuadas como fuente alternativa de este ácido graso. La biomasa microbiana, como fuente compleja, no solo de DHA sino de otros macro y micronutrientes, no presentó ningún efecto inhibidor en el crecimiento (Francis *et al.*, 2001) y tampoco fue beneficioso para la salud (Verschuere *et al.*, 2000; Rodríguez *et al.*, 2003), mas allá del beneficio nutritivo del DHA. Tampoco se observó ningún efecto negativo en su respuesta a la enfermedad como el descrito en alevines del salmón atlántico, que tras ser alimentados con dietas en las que el aceite de pescado se sustituyó por un thraustochytrido, se sometió a *Vibrio anguillarum* (Carter *et al.*, 2003). No se observaron diferencias en el nivel de incorporación de DHA alcanzado con la biomasa homogenizada y sin homogenizar, indicando su buena utilización nutritiva de las larvas de dorada en este estadio de crecimiento. Si se confirmase en otras especies y estadios de desarrollo, el homogenizado puede ser eliminado del

proceso de fermentación. Además la biomasa rica en DHA y sin homogenizar se encuentra naturalmente encapsulada y por tanto mejor protegida contra la oxidación.

La inclusión de las microalgas directamente en la dieta en lugar de la laboriosa implementación como enriquecedor en presas vivas, permite estudiar en detalle la nutrición de lípidos, sin interferencia con el metabolismo de las presas. Gracias al sencillo perfil de lípidos de las microalgas ensayadas en este trabajo, la deposición y transformación de los distintos ácidos en la dorada pudo ser estudiado en detalle mediante la sustitución completa de los aceites de pescado. Las microdietas suplementadas con microalgas no presentaron apenas EPA, un ácido graso esencial. A pesar de que las presas vivas son capaces de obtener este ácido graso a partir de la oxidación del DHA y DPA-6 de las algas (Barclay and Zeller, 1996; Ritar *et al.*, 2004), las larvas de *S. aurata* no mostraron apenas incorporación de EPA, indicando que tanto la oxidación a partir del DHA como su síntesis mediante la desaturación Δ^6 y Δ^5 (Tocher and Ghioni, 1999) para producir EPA es insuficiente. A pesar de que el contenido total de n-3 HUFA en larva entera alcanzado fue apropiado (2.5 % peso seco corporal), los requerimientos de EPA, que deberían representar la mitad de los requerimientos de DHA (Rodríguez *et al.*, 1998), no se cubrieron en el Experimento 2 (DHA: EPA 10:1). A la función d El EPA además de dotar de fluidez a la membrana en la que se incorpora, actúa como el precursor más importante de eicosanoides, moléculas involucradas en la respuesta inmune y de estrés de la dorada (Ganga *et al.*, 2005, 2006). Por ello, incluso cuando los requerimientos de DHA están cubiertos en la dieta, las deficiencias de EPA pueden causar un bajo creciemiento, supervivencia y resistencia al estrés (Liu *et al.*, 2002), tal y como se observa en el Experimento 2. Los niveles de ARA también fueron bajos en el presente experimento, aunque los niveles alcanzados en larvas fueron mayores a los ofrecidos por sus dietas, de acuerdo con lo descrito en rodaballo (Linares and Henderson, 1991). Además, tanto el contenido de ARA como el de DPA-6 de las larvas alimentadas con dietas de *Schizochytrium* fueron superiores que aquellas alimentadas con aceite de pescado o *C. cohnii*, indicando que el DPA-6 fue retro-convertido a ARA mediante oxidación de acuerdo con lo sugerido por Sargent *et al.* (1997). Por ello, además del interés de *Schizochytrium* como fuente de DHA, este alga podría contribuir indirectamente al contenido de ARA. A pesar de las pequeñas cantidades a las que se encuentra habitualmente el ARA en los peces, es un precursor importante de una familia diferente de eicosanoides que interactuan con los derivados del EPA regulando la respuesta de numerosos procesos fisiológicos (Bell *et al.*, 2003), controlando el crecimiento y la supervivencia larvaria (Bessonart *et al.*, 1999) y su respuesta al estrés (Koven *et al.*, 2001; Van Anholt *et al.*, 2004). Da-

do que fueron los bajos niveles de EPA en larva y no los de ARA los que coincidieron con los malos resultados obtenidos durante el Experimento 2, el efecto del ARA estuvo seguramente encubierto por el del EPA, como sugirió (Bessonart *et al.*, 1999). Por ello, la deficiencia de EPA observada, junto con otros factores asociados a las dietas algales, tales como los fosfolípidos, las vitaminas, los aminoácidos esenciales y los carbohidratos, así como el aceite de vegetal presentes en las dietas implementadas con el alga, pueden haber causado los malos resultados obtenidos con las microalgas heterótrofas durante el Experimento 2.

El ratio optimo de DHA: EPA para las larvas puede alcanzarse mediante la sustitución del aceite de pescado pero manteniendo la harina, la cual provee de EPA en sus lípidos. Por otro lado, el enriquecimiento de presas vivas con la biomasa de algas rica en DHA, permitiría alcanzar los ratios óptimos, ya que la *Artemia* y los rotíferos son capaces de convertir el DHA a EPA (Evjemo *et al.*, 2001; Ritar *et al.*, 2004). Diferentes larvas, incluyendo las de *S. aurata*, fueron alimentadas usando estas microalgas como enriquecedor (Koven *et al.*, 2001; Harel *et al.*, 2002). Sin embargo, la sustitución del aceite de pescado por la biomasa algal, a pesar de que ofrece una excelente fuente de DHA, no permite la sustitución completa de los aceites derivados de la pesca debido a su bajo contenido en EPA, indicando que se deberían implementar también fuentes de EPA con el objetivo de obtener un ratio óptimo de DHA: EPA en larvas. Varias microalgas, entre ellas *Odontella aurita*, *Monodus subterraneus* o *Nitzschia laevis*, así como el hongo (*Mortierella alpina*) poseen altas cantidades de EPA (Wen and Chen, 2005), sin embargo, el cultivo de estos organismos tendría que evolucionar hasta alcancar las productividades alcanzadas con el DHA en *Schizochytrium*. La posibilidad de desarrollar tecnologías de cultivo heterotróficas podría disminuir el costo de producción desde más de 20 US \$ kg^{-1} en cultivo fototrópico a menos de 5 US \$ kg^{-1} (Gladue and Maxey, 1994), permitiendo una producción industrial factible. Mientras tanto, estas microalgas ricas en DHA pueden sustituir el aceite de pescado presente en las microdietas para larvas, y también para alevines de dorada (Atalah *et al.*, in press) o parr de salmón atlántico (Miller *et al.*, in press). Su implementación en estadios nutritivamente menos exigentes, como los juveniles o adultos, dependerá del coste de producción de las algas y su competitividad con respecto a los lípidos derivados del pescado.

En conclusión, la biomasa sin homogenizar de las microalgas heterótrofas pueden emplearse eficientemente como fuentes de DHA en microdietas para *S. aurata*. En concreto, se recomienda la implementación de *Schizochytrium* sp. frente a *C. cohnii*, debido a su rápido crecimiento y a su capacidad para aumentar los niveles de ARA en larvas. Con todo, ofrecer a la larva con un ratio óptimo de DHA: EPA en ausencia de aceites derivados de la

pesca, requeriría desarrollar y experimentar otras fuentes de EPA como las heterotróficas.

Bibliografía

Abe, E., Hayashi, Y., Hama, Y., Hayashi, M., Inagaki, M. and Ito, M. (in press). A novel phosphatidylcholine which contains pentadecanoic acid and *sn*-1 and docosahexaenoic acid at *sn*-2 in *Schizochytrium* sp. F26-b. *J Biochem*, DOI 10.1093/jb/mvj145.

Ackman, R.G., Jangaard, P.M., Hoyle, R.J. and Brokerhoff, H. (1964) Analyses of the fatty acids produced by the diatom *Skeletonema costatum*. *J Fish Res Board Can*, 21:747–756.

Apromar (2006) Informe anual: La acuicultura marina de peces en España. Technical report, Asociación Empresarial de Productores de Cultivos Marinos www.apromar.es.

Apt, K.E. and Behrens, P.W. (1999) Commercial developments in microalgal biotechnology. *J Phycol*, 35:215–226.

Atalah, E. Hernández-Cruz, C.M., Izquierdo, M.S., Rosenlund, G., Caballero, M.J., Valencia, A. and Robaina, L. (In press) Two microalgae *Crypthecodinium cohnii* and *Phaeodactylum tricornutum* as alternative source of essential fatty acids in starter feeds for seabream (*Sparus aurata*). *Aquaculture*, DOI: 10.1016/j.aquaculture.2007.04.009.

Bailey, R.B., DiMasi, D., Hansen, J.M., Mirrasoul, P.J., Ruecker, C.M., Veeder III, G.T. and Kaneko, T. (2003) Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermentors. United States Patent: 09,771,352, *Martek Biosciences Corporation* Boulder, Colorado, pp. 1–12.

Barclay, W., Weaver, C. and Metz, J. (2005) Development of a docosahexaenoic acid production technology using *Schizochytrium*: a historical perspective. In: Ratledge, C. and Cohen, Z. (Eds.) Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 36–49.

Barclay, W. and Zeller, S. (1996) Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. *J World Aquacult Soc*, 27(3):314–322.

Barclay, W.R., Meager, K.M. and Abril, J.R. (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J Appl Phycol*, 6:123–129.

Barclay, W.R., Terry, K.L., Nagle, N.J., Weissman, J.C. and Goebel, R.P. (1987) Potential of new strains of marine and inland saline-adapted microalgae for aquaculture. *J World Aquacult Soc*, 18:218–228.

Battaglene, S.C., Morehead, D.T., Cobcroft, J.M., Nichols, P.D., Brown, M.R. and Carson, J. (2006) Combined effects of feeding enriched rotifers and antibiotic addition on performance of striped trumpeter (*Latris lineata*) larvae. *Aquaculture*, 251(1–2):456–471.

Beach, D.H., Harrington, G.W., Gellerman, J.L., Schlenk, H. and George G. Holz, J. (1974) Biosynthesis of oleic acid and docosahexaenoic acid by a heterotrophic marine dinoflagellate *Crypthecodinium cohnii*. *Biochim Biophys Acta*, 369:16–24.

Bell, J.G., Mc Evoy, L.A., Estévez, A., Shields, R.J. and Sargent, J.R. (2003) Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture*, 227(1–4):211–220.

Bell, J.G., Torstensen, B. and Sargent, J.R. (2005) Replacement of marine fish oils with vegetable oils in feeds for farmed salmon. *Lipid Tech*, 17:7–11.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C. and Sargent, J.R. (1995) Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids*, 30(5):443–449.

Beney, L. and Gervais, P. (2001) Influence of the fluidity of the membrane on the response of organisms to environmental stresses. *Appl Microbiol Biotech*, 57:34–42.

Benítez-Santana, T., Masuda, R., Juarez-Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M. and Izquierdo, M.S. (2006) Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. *Aquaculture*, 34(1):100–117.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M. and Fernández-Palacios, H. (1999) Effect of dietary arachidonic acid levels on growth and survival of gilthead sea bream (*Sparus aurata*) larvae. *Aquaculture*, 127(1–4):265–276.

Bistrian, B.R. (2003) Clinical aspects of essential fatty acid metabolism: Jonathan Rhoads lecture. *J Parent Ent Nutr*, 27(3):168–175.

Blair, T., Castell, J., Neil, S., D'Abramo, L., Cahu, C.L., Harmon, P. and Ogunmoye, K. (2003) Evaluation of microdiets versus live feeds on growth, survival and fatty acid composition of larval haddock (*Melanogrammus aeglefinus*). *Aquaculture*, 225(1–4):451–461.

Borowitzka, M.A. (1997) Microalgae for aquaculture: opportunities and constraints. *J Appl Phycol*, 9(5):393–401.

Boulton, C.A. and Ratledge, C. (1984) *Cryptococcus terricolus*, an oleaginous yeast re-appraised. *Appl Microbiol Biotech*, 20(1):72–76.

Brenna, J.T. (2002) Efficiency of conversion of α-linolenic acid to long chain n-3 fatty acids in man. *Curr Opin Clin Nutr*, 5(2):127–132.

Brenner, R.R. (2003) Hormonal modulation of Δ^6 and Δ^5-desaturases: case of diabetes. *Prostag Leukotr Ess*, 68(2):151–162.

Broadhurst, C.L. and Wang, Y. (2002) Brain-specific lipids from marine, lacustrine, or terrestrial food resources: potential impact on early African *Homo sapiens*. *Comp Biochem Phys B*, 131(4):653–673.

Brown, M.F. (1994) Modulation of rhodopsin function by properties of the membrane bilayer. *Chem Phys Lipids*, 73(1–2):159–180.

Brugére, C. and Ridler, N. (2004) Global aquaculture outlook in the next decades: an analysis of national aquaculture production forecasts 2030. *Fisheries Circular 1001*, FAO, Rome.

Burdge, G.C. and Wootton, S.A. (2002) Conversion of α-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *Brit J Nutr*, 88(4):411–420.

Burgess, J.R., Stevens, L., Zhang, W. and Peck, L. (2000) Long-chain polyunsaturated fatty acids in children with attention-deficit hyperactivity disorder. *Am J Clin Nutr*, 71(1):327–330.

Buzzi, M., Henderson, R.J. and Sargent, J.R. (1997) Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. *Comp Biochem Phys B*, 116(2):263–267.

Cahu, C.L., Zambonino-Infante, J.L. and Takeuchi, T. (2003) Nutritional components affecting skeletal development in fish larvae. *Aquaculture*, 227(1–4):254–258.

Calder, P.C. (2002) Dietary modification of inflammation with lipids. *P Nutr Soc*, 61(3):345–358.

Calder, P.C., Yaqoob, P., Thies, F., Wallace, F.A. and Miles, E.A. (2002) Fatty acids and lymphocyte functions. *Brit J Nutr*, 87(1):31–48.

Carter, C.G., Bransden, M.P., Lewis, E.T. and Nichols, P.D. (2003) Potential of thraustochytrids to partially replace fish oil in Atlantic salmon feeds. *Mar Biotechnol*, 5:480–492.

Cavalier-Smith, T., Allsopp, M.T.E.P. and Chao, E.E. (1994) Thraustochytrids are chromists, not fungi: 18S rRNA signatures of heterokonta. *Philos T Royal Soc B*, 348(1318):387–397.

Cheng, A.T.Y. (1998) How to make oxygen economical for fermentation. In: Pharmaceutical Ingredients Worldwide, *CPHI press*, Amsterdam, Netherlands, pp. 1–12.

Chen, C. and Bazan, N.G. (2005) Lipid signaling: sleep, synaptic plasticity, and neuroprotection. *Prost Oth Lipid M*, 77:65–76.

Cleland, L.G., James, M.J. and Proudman, S.M. (2003) The role of fish oils in the treatment of rheumatoid arthritis. *Drugs*, 63(9):845–853.

Cole, G.M., Lim, G.P., Yang, F., Teter, B., Begum, A., Ma, Q., Harris-White, M.E. and Frautschy, S.A. (2005) Prevention of Alzheimer's disease: ω-3 fatty acid and phenolic anti-oxidant interventions. *Neurobiol Aging*, 26(1):133–136.

Colwell, R.R. (1997) Microbial biodiversity and biotechnology. In: Colwell (Ed.), Biodiversity II: understanding and protecting our biological resources. *Joseph Henry Press of the National Academy Press*, Washington DC., pp. 279–287.

Connor, W.E. (2000) Importance of n-3 fatty acids in health and disease. *Am J Clin Nutr*, 71(1):171–175.

Copeman, L.A., Parrish, C.C., Brown, J.A. and Harel, M. (2002) Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder

(*Limanda ferruginea*): a live food enrichment experiment. *Aquaculture*, 210(1):285–304.

Chouinard, P.Y., Girard, V. and Brisson, G.J. (1998) Fatty acid profile and physical properties of milk fat from cows fed calcium salts of fatty acids with varying unsaturation. *J Dairy Sci*, 81:471–481.

Crawford, M.A., Bloom, M., Broadhurst, C.L., Schmidt, W.F., Cunnane, S.C., Galli, C., Gehbremeskel, K., Linseisen, F., Lloyd-Smith, J. and Parkington, J. (1999) Evidence for the unique function of docosahexaenoic acid during the evolution of the modern hominid brain. *Lipids*, 34:39–47.

Cunnane, S.C. (2000) The conditional nature of the dietary need for polyunsaturates: a proposal to reclassify essential fatty acids as conditionally-indispensable or conditionally-dispensable fatty acids. *Brit J Nutr*, 84(6):803–812.

Cutts, C.J., Sawanboonchun, J., Mazorra de Quero, C. and Bell, J.G. (2006) Diet-induced differences in the essential fatty acid (EFA) compositions of larval Atlantic cod (*Gadus morhua* L.) with reference to possible effects of dietary EFAs on larval performance. *ICES J Mar Sci*, 63(2):302–310.

de Swaaf, M.E., Pronk, J.T. and Sijtsma, L. (2003) Fed-batch cultivation of the docosahexaenoic-acid-producing marine alga *Crypthecodinium cohnii* on ethanol. *Appl Microbiol Biotechnol*, 61(1):40–43.

de Swaaf, M.E., de Rijk, T.C., van der Meer, P., Eggink, G. and Sijtsma, L. (2003) Analysis of docosahexaenoic acid biosynthesis in *Crypthecodinium cohnii* by C-13 labelling and desaturase inhibitor experiments. *J Biotech,* 103:21–29.

de Swaaf, M.E., Sijtsma, L. and Pronk, J.T. (2003b) High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga *Crypthecodinium cohni*. *Biotech Bioeng*, 81: 666–672.

Emken, E.A., Adolf, R.O. and Gulley, R.M. (1994) Dietary linoleic acid influences desaturation and acylation of deuterium-labelled linoleic and linolenic acids in young adult males. *Biochim Biophys Acta*, 1213(3):277–288.

Eroshin, V.K., Dedyukhina, E.G., Satroutdinov, A.D. and Chistyakova, T.I. (2002) Growth-coupled lipid synthesis in *Mortierella alpina* LPM301, a producer of arachidonic acid. *Microbiology*, 71(2):169–172.

Evans, C.T. and Ratledge, C. (1983) A comparison of the oleaginous yeast, *Candida curvata*, grown on different carbon-sources in continuous and batch culture. *Lipids*, 18(9):623–629.

Evjemo, J.O., Danielsen, T.L. and Olsen, Y. (2001) Losses of lipid, protein and n-3 fatty acids in enriched *Artemia franciscana* starved at different temperatures. *Aquaculture*, 193(1–2):65–80.

Fan, K.W., Chen, F., Jones, E.B.G. and Vrijmoed, L.L.P. (2001) Eicosapentaenoic and docosahexaenoic acids production by and okara-utilising potential of thraustochytrids. *J Indust Microbiol Biotech*, 27:199–202.

FAO (2004) Collation, analysis and dissemination of global and regional fishery statistics. Fi programme websites http://www.fao.org/figis/ [Accessed Dec 15 2006]., Fishery Information, Data and Statistics Unit (FAO-FIDI), Rome, pp. 144.

FAO/WHO (1994) Fats and oils in human nutrition. Joint expert consultation. Food and Nutrition, 57, (FAO/WHO) World Health Organisation, Rome, pp 120.

Faulk, C.K. and Holt, G.J. (2005) Advances in rearing cobia *Rachycentron canadum* larvae in recirculating aquaculture systems: live prey enrichment and greenwater culture. *Aquaculture*, 249(1–4):231–243.

Francis, G., Makkar, H.P.S. and Becker, K. (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199:197–227.

Franklin, S.T., Martin, K.R., Baer, R.J., Schingoethe, D.J. and Hippen, A.R. (1999) Dietary marine algae (*Schizochytrium* sp.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J Nutr*, 129(11):2048–2054.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S. (2005) Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). *Comp Biochem Physiol B*, 142(4):410–418.

Ganga, R., Tort, L., Acerete, L., Montero, D. and Izquierdo, M.S. (2006) Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*. *J Endocrin*, 190:39–45.

Ganuza, E. and Izquierdo, M.S. (2007) Lipid accumulation in *Schizochytrium* G13/2S produced in continuous culture. *Appl Microbiol Biotechnol*, 76(5):985–990.

Ganuza, E., Anderson, A.J. and Ratledge, C. (in press) High-cell-density cultivation of *Schizochytrium* in ammonium-pH-auxostat fed-batch system. *Biotechnol Lett*.

Gerster, H. (1998) Can adults adequately convert α-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int J Vit Nutr Res*, 68(3):159–173.

Gill, C.O., Hall, M.J. and Ratledge, C. (1977) Lipid-accumulation in an oleaginous yeast (*Candida* 107) growing on glucose in single-stage continuous culture. *Appl Environ Microb*, 33(2):231–239.

Giraud, M.N., Motta, C., Boucher, D. and Grizard, G. (2000) Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. *Hum Reprod*, 15(10):2160–2164.

Gladue, R.M. and Maxey, J.E. (1994) Microalgal feeds for aquaculture. *J Appl Phycol*, 6(6):131–141.

Granger, L.M., Perlot, P., Goma, G. and Pareilleux, A. (1993) Effect of various nutrient limitations on fatty-acid production by *Rhodotorula glutinis*. *Appl Microbiol Biotech*, 38(6):784–789.

Hall, M.J. and Ratledge, C. (1977) Lipid-accumulation in an oleaginous yeast (*Candida* 107) growing on glucose under various conditions in a one-stage and two-stage continuous culture. *Appl Environ Microbiol*, 33:577–584.

Hansen, H.S., Jensen, B. and von Wettstein-Knowles, P. (1986) Apparent in vivo retroconversion of arachidonic acid to linoleic acid. *Biochim Biophys Acta*, 878(2):284–287.

Harel, M., Koven, W., Lein, I., Bar, Y., Behrens, P., Stubblefield, J., Zohar, Y. and Place, A.R. (2002) Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs. *Aquaculture*, 213(2):347–362.

Henderson, R.J., Mackinlay, E.E., Hodgsona, P.A. and Hardwood, J.L. (1990) Differential effects of the substituted pyridazinone herbicide Sandoz 9785 on lipid composition and biosynthesis in photosynthetic and non-photosynthetic marine microalgae. II. Fatty acid composition. *J Exp Bot*, 41:729–736.

Henderson, R.J. and Mackinlay, E.E. (1991) Polyunsaturated fatty-acid metabolism in the marine dinoflagellate *Crypthecodinium cohnii*. *Phytochemistry*, 30:1781–1787.

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawa, S. and Shimizu, S. (1998) Enhancement of arachidonic acid production by *Mortierella alpina* 1S–4. *J Am Oil Chem Soc*, 75(11):1471–1586.

Hites, R.A., Foran, J.A., Carpenter, D.O., Hamilton, M.C., Knuth, B.A. and Schwager, S.J. (2004) Global assesment of organic contaminants in farmed salmon. *Science*, 303:226–229.

Hong, S., Gronert, K., Devchand, P.R., Moussignac, R.L. and Serhan, N.C. (2003) Novel docosatrienes and 17s-resolvins generated from docosahexaenoic acid in murine brain, human blood and glial cells. *J Biol Chem*, 278(17):14677–14687.

Ibeas, C., Cejas, J.R., Fores, R., Badía, P., Gómez, T. and Hernández, A.L. (1997) Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream (*Sparus aurata*) juveniles. *Aquaculture*, 150(1–2):91–102.

Iijima, N., Tanaka, S. and Ota, Y. (1998) Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. *Fish Physiol Biochem*, 18(1):59–69.

Ingram, L.O., Chevalier, L.S., Gabba, E.J., Ley, K.D. and Winters, K. (1977) Propionate-induced synthesis of odd-chain-length fatty acids by *Escherichia coli*. *J Bacteriol*, 131(3):1023–1025.

Izquierdo, M.S. (1988) Essential fatty acid requirements for marine fish larvae. PhD thesis, La Laguna University, Spain, pp. 255.

Izquierdo, M.S. (1996) Essential fatty acid requirements of cultured marine fish larvae. *Aquacult Nutr*, 2(2):183–191.

Izquierdo, M.S. (2005) Essential fatty acid requirements in Mediterranean fish species. *Cah Options Mediterr*, 63:91–102.

Izquierdo, M.S., Fernández-Palacios, H. and Tacon, A.G.J. (2001) Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture*, 197(1–4):25–42.

Izquierdo, Montero, D., Robaina, L., Caballero, M.J., Rosenlund, G. and Ginés, R. (2005) Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. *Aquaculture*, 250:431–444.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L. and Rosenlund, G. (2003) Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. *Aquacult Nutr*, 9(6):397–407.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernández-Cruz, C. (2000) Recent advances in lipid nutrition in fish larvae. *Fish Physiol Biochem*, 22:97–107.

James, M.J., Ursin, V.M. and Cleland, L.G. (2003) Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am J Clin Nutr*, 77(5):1140–1145.

Jeffrey, B.G., Weisinger, H.S., Neuringer, M. and Mitchell, D.C. (2001) The role of docosahexaenoic acid in retinal function. *Lipids*, 36(9):859–871.

Josupeit, H. (2006) Fish oil production disappointing. Fish info network market report, FAO Globefish, Rome, pp. 2.

Kemperman, R.F., Veurink, M., van der Wal, T., Knegtering, H., Bruggeman, R., Fokkema, M.R., Kema, I.P., Korf, J. and Muskiet, F.A. (2006) Low essential fatty acid and B-vitamin status in a subgroup of patients with schizophrenia and its response to dietary supplementation. *Prostag Leukotr Ess*, 74(2):75–85.

Kendrick, A. and Ratledge, C. (1992) Lipid formation in the oleaginous mold *Entomophthora exitalis* grown in continuous culture. Effects of growth-rate, temperature and dissolved-oxygen tension on polyunsaturated fatty-acids. *Appl Microbiol Biotech*, 37(1):18–22.

Kennedy, M.J., Reader, S.L. and Davies, R.J. (1993) Fatty acid production characteristics of fungi with particular emphasis on γ-linolenic acid production. *Biotech Bioeng*, 42(5):625–634.

Khaware, R.K., Koul, A. and Prasad, R. (1995) High membrane fluidity is related to NaCl stress in *Candida membranefaciens*. *Biochem Mol Biol Int*, 4:875–880.

Kingsella, J.E., Lokesh, B., Broughton, S. and Whelan, J. (1990) Dietary polyunsaturated fatty acids and eicosanoids: potential effects on the modulation of inflamatory and immune cells: an overview. *Nutrition*, 6(1):24–62.

Klein, H.P. and Volkmann, C.M. (1975) Factors affecting palmitoylcoenzyme A desaturase of *Saccharomyces cerevisiae*. *J Bacteriol*, 124:718–723.

Koven, W.M., Kissil, G.W. and Tandler, A. (1989) Lipid and n-3 requirement of *Sparus aurata* larvae during starvation and feeding. *Aquaculture*, 79:185–191.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P. and Tandler, A. (2001) The effect of dietary arachidonic acid (20:4 n-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. *Aquaculture*, 193(1–2):107–122.

Koven, W., Kolkovski, S., Hadas, E., Gamsiz, K. and Tandler, A. (2001) Advances in the development of microdiets for gilthead seabream, *Sparus aurata*: a review. *Aquaculture*, 194(1–2):107–121.

Kris-Etherton, P.M., Harris, W.S. and Appel, L.J. (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*, 106(1):2747–2757.

Kris-Etherton, P.M., Taylor, D.S., Yu-Poth, S., Huth, P., Moriarty, K., Fishell, V., Hargrove, R.L., Zhao, G. and Etherton, T.D. (2000) Polyunsaturated fatty acids in the food chain in the United States. *Am J Clin Nutr*, 71(1):179–188.

Kyle, D.J. and Gladue, R.M. (1993) Eicosapentaenoic acids and methods for their production. United States Patent 5244921, *Martek Corporation*, Columbia, pp. 60.

Lewis, N.M., Seburg, S. and Flanagan, N.L. (2000) Enriched eggs as a source of n-3 polyunsaturated fatty acids for humans. *Poultry Sci*, 79(7):971–974.

Lichtenstein, A.H. (2003) Dietary fat and cardiovascular disease risk: quantity or quality? *J Women Health Gen B*, 12(2):109–114.

Linares, F. and Henderson, R.J. (1991) Incorporation of 14C-labelled polyunsaturated fatty acids by juvenile turbot, *Scophthalmus maximus* (L.) *in vivo*. *J Fish Biol*, 38:335–347.

Liu, J., Caballero, M.J., Izquierdo, M.S., El-Sayed-Ali, T., Hernández-Cruz, C.M., Valencia, A. and Fernández-Palacios, H. (2002) Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead seabream *Sparus aurata*. *Fisheries Sci*, 68(6):1165–1172.

Lopes da Silva, T., Mendes, A., Mendes, R.L., Calado, V., Alves, S.S., Vasconcelos, J.M.T. and Reis, A. (2006) Effect of n-dodecane on *Crypthecodinium cohnii* fermentations and DHA production. *J Ind Microbiol Biot*, 3(6):408–16.

Madsen, L., Rustan, A.C., Vaagenes, H., Berge, K., Dyroy, E. and Berge, R.K. (1999) Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate reference. *Lipids*, 34(9):951–963.

Masuda, R. (2003) The critical role of docosahexaenoic acid in marine and terrestrial ecosystems: from bacteria to human behaviour. In Browman, H.I. and Skiftesvik, A.B., (Eds), *The Big Fish Bang*, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, Bergen, Norway, pp. 249–256.

Masuda, R., Takeuchi, T., Tsukamoto, K., Ishizaki, Y., Kanematsu, M. and Imaizum, K. (1998) Critical involvement of dietary docosahexaenoic acid in the ontogeny of schooling behaviour in the yellowtail. *J Fish Biol*, 53(3):471–484.

Mc Evoy, L.A., Naess, T., Bell, J.G. and Lie, Ø. (1998) Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglossus hippoglossus*) fed enriched *Artemia*: a comparison with fry fed wild copepods. *Aquaculture*, 163(3–4):237–250.

Mc Evoy, L.A., Navarro, J.C., Bell, J.G. and Sargent, J.R. (1995) Autoxidation of oil emulsions during the *Artemia* enrichment process. *Aquaculture*, 134(1–2):101–112.

Meesters, P.A.E.P., Huijberts, G.N.M. and Eggink, G. (1996) High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Appl Microbiol Biotech*, 45:575–579.

Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V. and Browse, J. (2001) Production of polyunsaturated fatty

acids by polyketide synthases in both prokaryotes and eukaryotes. *Science*, 293:290–293.

Meyer, F. and Bolch, K. (1963) Effect of temperatures on the enzymatic synthesis of unsaturated fatty acids in *Torulopsis utilis*. *Biochim Biophys Acta*, 77:671–673.

Miller, M.R., Nichols, P.D. and Carter, C.G. (in press) Replacement of fish oil with thraustochytrid *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. *Comp Biochem Physiol A*, DOI 17588797.

Montero, D., Socorro, J., Tort, L., Caballero, M.J., Robaina, L.E., Vergara, J.M. and Izquierdo, M.S. (2004) Glomerulonephritis and immunosuppression associated with dietary essential fatty acid deficiency in gilthead sea bream, *Sparus aurata* L., juveniles. *J Fish Dis*, 27(5):297–306.

Moreton, R.S. (1988) Physiology of lipid accumulating yeasts. In: Moreton, R.S. (Eds.) Single cell oils, *The Bath Press*, Essex, UK, pp. 1–28.

Mourente, G. (2003) Accumulation of DHA (docosahexaenoic acid; 22:6n-3) in larval and juvenile fish brain. In: Browman, H.I. and Skiftesvik, A.B. (Eds), *The Big Fish Bang*, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, Bergen, Norway, pp. 139–148.

Mourente, G. and Odriozola, J.M. (1990) Effect of broodstock diets on lipid classes and their fatty acid composition in eggs of gilthead sea bream (*Sparus aurata* l.). *Fish Phys Bioch*, 8(2):93–101.

Müller-Navarra, D.C., Brett, M.T., Liston, A.M. and Goldman, C.R. (2000) A highly- unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature*, 403:74–77.

Müller-Navarra, D.C., Brett, M.T., Sangkyu, P., Chandra, S., Ballantyne, A.P., Zorita, E. and Goldman, C.R. (2004) Unsaturated fatty acid content in seston and tropho-dynamic coupling in lakes. *Nature*, 427(6969):69–72.

Nakamura, M.T. and Nara, T.Y. (2003) Essential fatty acid synthesis and its regulation in mammals. *Prostag Leukotr Ess*, 68(2):145–150.

Nakayama, H., Mitsui, T., Nishihara, M. and Kito, M. (1980) Relation between growth temperature of *E. coli* and phase transition temperatures of its cytoplasmic and outer membranes. *Biochim Biophys Acta*, 601(1):1–10.

Navarro, J.C., Amat, F. and Sargent, J.R. (1993) The lipids of the cysts of freshwater- and marine-type *Artemia*. *Aquaculture*, 109(3–4):327–336.

Naylor, R.L., Goldburg, J.H., Primaver, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenko, J., Mooney, H. and Troell, M. (2000) Effect of aquaculture in world fish supplies. *Nature*, 405:1017–1024.

Nettleton, J.A. (1991) Omega-3 fatty acids: comparison of plant and seafood sources in human nutrition. *J Am Diet Assoc*, 91(3):331–337.

O'Mara, C.J. (1998) U.S. oil seed industry looks at trade issues. *Inform*, 9:132–136.

Owen, J.M., Adron, J.W., Middleton, C., and Cowey, C.B. (1975). Elongation and desaturation of dietary fatty acids in turbot *Scophtalmus maximus* L., and rainbow trout, *Salmo gairdnerii*. *Lipids*, 10(9):528–531.

Park, H.G., Puvanendran, V., Kellett, A.P., Christopher, C. and Brown, J.A. (2006) Effect of enriched rotifers on growth, survival, and composition of larval Atlantic cod (*Gadus morhua*). *ICES J Mar Sci*, 63(2):285–295.

Peyou-Ndi, M.M., Watts, J.L. and Browse, J. (2000) Identification and characterization of an animal Δ-fatty acid desaturase gene by heterologous expression in *Saccharomyces cerevisiae*. *Arch Biochem Biophys*, 376(2):399–408.

Qiu, X., Hong, H.P. and MacKenzie, S.L. (2001) Identification of a Δ^4 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(2):31561–31566.

Rabinovich, A.R. and Ripatti, P.O. (1991) On the conformational, physical properties and functions of polyunsaturated acyl chains. *Biochim Biophys Acta*, 1085(1):53–62.

Rapoport, S.I. and Bosetti, F. (2002) Do lithium and anticonvulsants target the brain arachidonic acid cascade in bipolar disorder? *Arch Gen Psychiat*, 59(7):592–596.

Ratledge, C. (1992) Microbial lipids: commercial realities or academic curiosities. In: Ratledge, C. (Ed), Industrial applications of single cell oils. *AOCS press*, Champaign, Illinois, pp. 1–15.

Ratledge, C. (2002a) Micro-organisms as sources of polyunsaturated fatty acids. In: Gunstone, F.D. (Ed), Structured and modified lipids, *Marcel Dekker, Inc.*, Basel, New York, pp. 351–400.

Ratledge, C. (2002b) Regulation of lipid accumulation in oleaginous micro-organisms. *Biochem Soc Trans*, 30:1047–1050.

Ratledge, C. (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, 86:807–815.

Ratledge, C. and Wynn, J.P. (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbiol*, 51:1–51.

Ristic, V. and Ristic, G. (2003) Role and importance of dietary polyunsaturated fatty acids in the prevention and therapy of atherosclerosis. *Medicinski Pregled*, 56(1–2):50–53.

Ritar, A.J., Dunstan, G.A., Nelson, M.M., Brown, M.R., Nichols, P.D., Thomas, C.W., Smith, E.G., Crear, B.J. and Kolkovski, S. (2004) Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. *Aquaculture*, 239(1–4):351–373.

Robin, J.H. and Peron, A. (2004) Consumption vs. deposition of essential fatty acids in gilthead sea bream (*Sparus aurata*) larvae fed semi-purified diets. *Aquaculture*, 238(1–4):283–294.

Rodríguez, C., Izquierdo, M.S., Lorenzo, A., and Fernández-Palacios, H. (1994a) The effect of image HUFA proportions in diets for gilthead seabream (*Sparus aurata*) larval culture. *Aquaculture*, 124(1–4):124–284.

Rodríguez, C., Pérez, J.A., Badía, P., Izquierdo, M.S., Fernández-Palacios, H. and Hernández, A.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(1–2):9–23.

Rodríguez, C., Pérez, J.A., Díaz, M., Izquierdo, M.S., Fernández-Palacios, H. and Lorenzo, A. (1997) Influence of the EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. *Aquaculture*, 150(1–2):77–89.

Rodríguez, C., Pérez, J.A., Lorenzo, A., Izquierdo, M.S. and Cejas, J.R. (1994b) N-3 HUFA requirement of larval gilthead seabream (*Sparus aurata*) when using high levels of eicosapentaenoic acid. *Comp Biochem Physiol A*, 107(4):693–698.

Rodríguez, A., Esteban, M.A. and Meseguer, J. (2003) A mannose-receptor is possibly involved in the phagocytosis of *Saccharomyces cerevisiae* by seabream (*Sparus aurata* L.) leucocytes. *Fish Shellfish Immu*, 14(5):375–388.

Roux, M.P., Kock, J.L.F., du Preez, J.C. and Botha, A. (1995) The influence of dissolved oxygen tension on the production of cocoa butter equivalents and γ-linolenic acid by *Mucor circinelloides*. *Syst Appl Microbiol*, 18(3):329–334.

Saito, H., Ishihara, K. and Murase, T. (1997) The fatty acid composition in tuna (bonito, *Euthynnus pelamis*) caught at three different localities from tropics to temperate. *J Sci Food Agric*, 73(1):53–59.

Salhi, M., Kolkovski, S., Izquierdo, M.S. and Tandler, A. (1995) Inclusion of lecithin on polar or neutral lipids high in n-3 HUFA in microdiets for gilthead seabream (*Sparus aurata*) larvae. In: Lavens, P., Jaspers, E., and Roelants, I., (Eds), *Larvi'95*, EAS Special publication, Gent, France, 26:184–187.

Sanders, T.A. (2000) Polyunsaturated fatty acids in the food chain in Europe. *Am J Clin Nutr*, 71(1):176–178.

Sargent, J.R., Mc Evoy, L.A. and Bell, J.G. (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture*, 155(1):117–127.

Sargent, J.R., Mc Evoy, L.A., Estévez, A., Bell, J.G., Bell, M., Henderson, J. and Tocher, D.R. (1999) Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture*, 179(1):217–229.

Sargent, J.R. and Tacon, A.G.J. (1999) Development of farmed fish: a nutritionally necessary alternative to meat. *P Nutr Soc*, 58(2):377–383.

Sargent, J.R., Tocher, D.R. and Bell, J.G. (2003) The lipids In: Halver, J. and Hardy, R. (Eds), Fish Nutrition, *Academic press*, San Diego, California, 3^{th} edition, pp. 181–260.

Serhan, N.C., Hong, S., Gronert, K., Colgan, S.P., Devchand, P.R., Mirick, G. and Moussignac, R.L. (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transfirmation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med*, 196(8):1025–1037.

Shepherd, C.J., Pike, I.H. and Barlow, S.M. (2005) Sustainable feed resources of marine origin. In: *Aquaculture Europe 2005*, EAS Special Publication, 35:59–66.

Shields, R.J. and Irwin, S. (1998) Preliminary evaluation of the *Schizochytrium*-based products, Algamac-3010 and Schizotein, as diet enrichments for Atlantic halibut larvae. Technical report, *Aquafauna BioMarine Inc.*, Algyll, UK.

Shirasaka, N., Hirai, Y., Nakabayashi, H. and Yoshizumi, H. (2005) Effect of cyanocobalamin and p-toluic acid on the fatty acid composition of *Schizochytrium limacinum* (Thraustochytriaceae, Labyrinthulomycota). *Mycoscience*, 46:358–363.

Sijtsma, L. and de Swaaf, M.E. (2004) Biotechnological production and applications of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. *Appl Microbiol Biotech*, 64(2):146–153.

Sijtsma, L., Springer, J., Meesters, P.A.E.P., de Swaaf, M.E. and Eggink, G. (1998) Recent advances in fatty acid synthesis in oleaginous yeast and microalgae. *Recent Res Dev Microbiol*, 2:219–232.

Simopoulos, A.P. (1999) Essential fatty acids in health and chronic disease. *Am J Clin Nutr*, 70(3):560–569.

Simopoulos, A.P. (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr*, 21(3):495–505.

Singh, A., Wilson, S. and Ward, O.P. (1996) Docosahexaenoic acid (DHA) production by *Thraustochytrium* sp. ATCC 20892. *World J Microbiol Biotech*, 12(1):76–81.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M. and Herndl, G.J. (2006) Microbial diversity in the deep sea and the underexplored rare biosphere. *World J Microbiol Biotech*, 103(32):12115–12120.

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

Sontrop, J. and Campbell, M.K. (2006) Omega-3 polyunsaturated fatty acids and depression: a review of the evidence and a methodological critique. *Prev Med*, 42(1):4–13.

Sprecher, H. (2002) The roles of anabolic and catabolic reactions in the synthesis and recycling of polyunsaturated fatty acids. *Prostag Leukotr Ess*, 67(2–3):79–83.

Sprecher, H.l., Luthria, D., Mohammed, B.S. and Baykousheva, S.P. (1995) Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J Lipid Res*, 36(12):2471–2477.

Staff reporter (2006) Global fish oil production down in 2005. *Food Production Daily*: www.foodproductiondaily.com.

Stillwell, W. and Wassall, S.R. (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chem Phys Lipids*, 126(1):1–27.

Tacon, A. (2004) Use of fish meal and fish oil in aquaculture: a global perspective. *Aq Res Cult Dev*, 1(1):3–14.

Terry, P.D., Rohan, T.E. and Wolk, A. (2003) Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. *Am J Clin Nutr*, 77(3):532–543.

Tocher, D.R., Agaba, M., Hastings, N. and Teale, A.J. (2003) Biochemical and molecular studies of the polyunsaturated fatty acid desaturation pathway in fish. In: Browman, H.I. and Skiftesvik, A.B. (Eds), *The Big Fish Bang*, Proceedings of the 26th Annual Larval Fish Conference, Institute of Marine Research, Bergen, Norway, pp. 211–227.

Tocher, D.R. and Ghioni, C. (1999) Fatty acid metabolism in marine fish: low activity of Δ^6 desaturation in gilthead sea bream (*Sparus aurata*) cells. *Lipids*, 34(5):433–440.

Tsai, P.S., Rao, G. and Bailey, J.E. (1995) Improvement of *Escherichia coli* microaerobic oxygen metabolism by *Vitreoscilla* hemoglobin: new insights from NAD(P)H fluorescence and culture redox potencial. *Biotechnol Bioeng*, 47(3):347–354.

Valero, E., Millán, C. and Ortega, J.M. (2001) Influence of oxygen addition during growth phase on the biosynthesis of lipids in *Saccharomyces cerevisiae* (M330-9) in enological fermentations. *J Biosci Bioeng*, 92:33–38.

Van Anholt, R.D., Spanings, F. A.T., Koven, W.M., Nixon, O., and Wendelaar Bonga, S.E. (2004) Arachidonic acid reduces the stress response of gilthead seabream *Sparus aurata* L. *J Exp Biol*, 207(19):3419–3430.

Van Pelt, C.K., Huang, M., Tschanz, C.L. and Brenna J.T. (1999) An octaene fatty acid, 4,7,10,13,16,19,22,25-octacosaoctaenoic acid (28:8n3), found in marine oils. *J Lipid Res*, 40:1501–1501.

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol R*, 64(4):655–671.

Vlaeminck, B., Fievez, V., Tamminga, S., Dewhurst, R.J., van Vuuren, A., De Brabander, D. and Demeyer, D. (2006) Milk odd- and branched-chain fatty acids in relation to the rumen fermentation pattern. *J Dairy Sci*, 89:3954–3964.

Voss, A., Reinhart, M., Sankarappa, S. and Sprecher, H. (1991) The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a Δ^4-desaturase. *J Biol Chem*, 266(30):19995–20000.

Wainwright, P.E. (2002) Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. *Proc Nutr Soc*, 61(1):61–69.

Wang, Y., Kim, H. and Weete, J. D. (2000) Docosahexaenoic production by *Thraustochytrium aureum*. In *91st AOCS Annual Meeting & Expo*, San Diego, California.

Ward, O.P. and Singh, A. (2005) Omega-3/6 fatty acids: alternative sources of production. *Process Biochem*, 40(12):3627–3652.

Watanabe, T. (1982) Lipid nutrition in fish. *Comp Biochem Physiol B*, 73(1):3–15.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S. and Kitajima, C. (1989) Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. *Nippon Suisan Gakk*, 55(9):1635–1640.

Watanabe, T. and Kiron, V. (1994) Prospects in larval fish dietetics. *Aquaculture*, 124(1–4):223–251.

Watanabe, T., Kitajimab, C. and Fujitab, S. (1983) Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture*, 34(1–2):115–.143.

Wen, Z. and Chen, F. (2005) Prospects for eicosapentaenoic acid production using microorganisms. In: Ratledge, C. and Cohen, Z. (Eds.), Single Cell Oils, *AOCS Press*, Champaign, Illinois, 1^{st} edition, pp. 138–181.

Whelan, J. (1996) Antagonistic effects of dietary arachidonic acid and n-3 polyunsaturated fatty acids. *J Nutr*, 126(4):1086–1091.

Yamane, Y., Higashida, K., Nakashimada, Y., Kakizono, T. and Nishio, N. (1997) Influence of oxygen and glucose on primary metabolism and astaxanthin production by *Phaffia rhodozyma* in batch and fed-batch cultures: kinetic and stoichiometric analysis. *Appl Environ Microb*, 63(11):4471–4478.

Yaguchi, T., Tanaka, S., Yokochi, T., Nakahara, T. and Higashihara, T. (1997) Production of high yields of docosahexaenoic acid by *Schizochytrium* sp. strain SR21. *J Am Oil Chem Soc*, 74:1431–1434.

Yavin, E., Brand, A. and Green, P. (2002) Docosahexaenoic acid abundance in the brain: a biodevice to combat oxidative stress. *Nutr Neurosc*, 5(3):149–157.

Zaslavskaia, L.A., Lippmeier, J.C., Shih, C., Ehrhardt, D., Grossman, A.R. and Apt, K.E. (2001) Trophic obligate conversion of an photoautotrophic organism through metabolic engineering. *Science*, 292(5524):2073–2075.

List of publications

Ganuza, E., Benítez-Santana, T., Atalah, E., Vega-Orellana, O., Ganga, R. and Izquierdo, M.S. (in press) *Crypthecodinium cohnii* and *Schizochytrium* sp. as potential substitutes to fisheries derived oils in *Sparus aurata* microdiets. *Aquaculture*.

Ganuza, E., Anderson, A.J. and Ratledge, C. (in press) High-cell-density cultivation of *Schizochytrium* in ammonium/pH-auxostat fed-batch system. *Biotechnol Lett*.

Ganuza, E. and Izquierdo, M.S. (2007) Lipid accumulation in *Schizochytrium* G13/2S produced in continuous culture. *Appl Microbiol Biotech*, 270(76):985-990.

Benítez-Santana, T., Masuda, R., Juárez-Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M. and Izquierdo, M.S. (2007) Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. *Aquaculture*, 264(1–4):408–417.

Atalah, E., Hernández-Cruz, C.M., Izquierdo, M.S., Rosenlund, G., Caballero, M.J., Valencia, A. and Robaina, L. (2007) Two microalgae *Crypthecodinium cohnii* and *Phaeodactylum tricornutum* as alternative source of essential fatty acids in starter feeds for seabream (*Sparus aurata*). *Aquaculture*, 270(1–4):178–185.

Ganuza, E. (2006) Cultivation of microorganisms rich in DHA as an alternative source to fish oil in aquafeeds. In: *Campus of Excellence* (nobel price awarded audience proceedings). Special Publication, Fundación Vitalia, Canary Islands, Spain.

Izquierdo, M.S., Benítez–Santana, T., Atallah, E., Ganuza, E., Roo, J. and Hernández–Cruz, C.M. (2006) Lipid nutrition and lipid sources for marine fish larvae. In *Aqua 2006*, The annual meeting of the world aquaculture society, Florence, Italy.

Ganuza, E., Ratledge, C., Anderson, A.J., Hopkins, S.A. and Izquierdo, M.S. (2005) Lipid accumulation in *Schizochytrium* G13/2S under continuous culture. In: Puhler, A. (Ed.), *Bringing genomes to life*, 12^{th} European Congress of Biotechnology, Copenhagen, Denmark. *J Biotechnol*, 118–189.

Ganuza, E., Ratledge, C., Anderson, A.J. and Izquierdo, M.S. (2005) High-cell-density cultivation of *Schizochytrium* sp. in ammonia/pH-auxostat fed-batch system. In: Kawai, H. (Ed.), 8^{th} *International Phycological Congress*, Durban, South Africa. *Phycologia*, 44:120–126.

Curriculum vitae

Eneko Ganuza Taberna was born on November 3^{th}, 1978 in Pamplona, Navarre. He obtained his Bachelor of Science in Oceanography by the University of Vigo, through EU exchange programmes. He studied at the Universidad de las Palmas de Gran Canaria, Universidade dos Açores (Portugal), Universidade de Vigo and at the Universidad de Cadiz. As an undergraduate student he developed a research study about the pot fisheries of the red mullet *Mullus surmuletus* for the Porto Abrigo Fisheries Cooperative (São Miguel, Açores). After graduation he began a postgraduate Course in Aquaculture with the Grupo de Investigación en Aquicultura (GIA) (Las Palmas, Spain) which was completed with short-courses on Fish Products Quality (Aqua TT) at the University College of Cork (Ireland) and Marine Algae Culture course at the CIHEAM (Zaragoza, Spain). In September 2003 he began his PhD studies at the Lipid Research Centre in the University of Hull studying the physiology of lipid accumulation microalgae under the supervision of Prof. Colin Ratledge and Dr. Alistair J. Anderson. The second part of the thesis research focused on the implementation of microbial lipid sources in aquaculture and was carried out as a member of the GIA in the Instituto Canario de Ciencias Marinas. The thesis has been structured and supervised by Prof. Marisol Izquierdo throughout the entire research period. The preliminary results were presented in Campus de Excelencia (Lanzarote, Spain), before a Nobel prize awarded audience.