MICROBIAL OILS AND FATTY ACIDS: EFFECT OF CARBON SOURCE ON DOCOSAHEXAENOIC ACID (C22:6 N-3, DHA) PRODUCTION BY THRAUSTOCHYTRID STRAINS

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ABSTRACT

Thraustochytrids are marine protists found throughout the world in estuarine and marine habitats. These microorganisms have attracted interest, because their lipids contain different long chain polyunsaturated fatty acids (PUFAs). Those able to produce docosahexaenoic acid (C22:6 n-3, DHA) are the most studied because of the physiological importance of this PUFA in human beings. Their heterotrophic cultivation offers several challenges since biomass, lipid content, and fatty acid profile are dependent on growth conditions. In this work the effects of C source and its concentration on DHA production by different thraustochytrid strains are reviewed. Results obtained by different investigators on the use of alternative and low cost nutrient sources for production of DHA by thraustochytrids are also presented.

Keywords: *Thraustochytrium*, *Schizochytrium*, docosahexaenoic acid, polyunsaturated fatty acids, lipids, carbon source, nitrogen source.

INTRODUCTION

Docosahexaenoic acid (C22:6 n-3, DHA), a highly unsaturated long chain fatty acid, is a major structural component in neural tissues and retina. It is considered essential for infants' nutrition, because it is involved in brain development. Dietary DHA has positive effect on several diseases such as hypertension, arthritis, atherosclerosis, depression, adult-onset diabetes mellitus, myocardial infarction, thrombosis, and some cancers (Horrocks and Yeo, 1999).

At present time, fatty fish from cold marine waters and fish oils are the main sources of DHA. In fish oils, both composition and quantity of polyunsaturated fatty acids (PUFAs) depend on the species, and time and geographical location of the capture. In addition, conditions of processing and refining can affect the quality of PUFAs in fish oils. PUFAs are naturally found with *cis* isomery (over 95%) to which all metabolic and structural functions are related.

Fish, just like human beings, have a low capability to synthesize long chain PUFAs, such as DHA, and rely on

phytoplankton, autotrophic bacteria and components in the zooplankton to obtain these molecules (Iwamoto and Sato 1986). Several species of marine bacteria, especially those found in cold and high pressure environments, accumulate lipids with high percentages of eicosapentaenoic acid (C20:5 n-3, EPA) and DHA (Nichols *et al*., 1993). Profile of the fatty acids synthesized by marine bacteria is very simple unlike the complex mixture found in fish oils and oils accumulated by phototrophic microalgae; the disadvantage of this microbial PUFA source is its low lipid yield (Nichols *et al*., 2002). Higher contents of PUFAs have been found in the biomass of different thraustochytrid strains isolated from estuarine and marine habitats throughout the world.

Thraustochytrids are marine protists that have been classified into the class Labyrinthulomycetes, a phylogenetic group in the stramenopile organisms, which consists of two well accepted families, i.e., Labyrinthulaceae and Thraustochytriaceae (Honda *et al*., 1999; Leipe *et al.,* 1994; Porter, 1989). The Thraustochytriaceae family is composed of seven genera: *Thraustochytrium*, *Schizochytrium*, *Ulkenia*, *Labyrinthuloides*, *Japonochytrium*, *Aplanochytrium* and *Althornia* (Porter, 1989). Combination of morphological and chemotaxonomic features such as PUFA profiles and carotenoid pigments can be used to distinguish at genus-level phylogenetic groups in the Labyrinthulomycetes (Yokoyama *et al*., 2007; Yokoyama and Honda, 2007). Six different PUFA profiles have been reported in thraustochytrids (Huang *et al*., 2003; Burja *et al*., 2006). These are: (1) DHA and docosapentaenoic acid (DPA, C22:5 n-6); (2) DHA, DPA, and EPA; (3) DHA and EPA; (4) DHA, DPA, EPA, and arachidonic acid (ARA, C20:4 n-6); (5) DHA, DPA, EPA, ARA, and docosatetraenoic acid (DTA, C22:4 n-6); and (6) DHA, EPA, and ARA.

Products described in this microorganisms include β-carotene, astaxanthin and canthaxanthin (Aki *et al*., 2003), squalene, a precursor of phytosterols (Jiang *et al*., 2004; Fan and Chen, 2007), phospholipids (Okuyama *et al*., 2007), sterols, and sulfated extracellular polysaccharides (Jain *et al*., 2005). Although all these products are demanded by pharmaceutical and food industries the most valued is DHA. This long chain PUFA is found in storage lipids, and cell membrane phospholipids in thraustochytrid cells (Morita *et al*., 2006). Up to now it is not clear why these protists produce DHA as storage lipids, although it is believed that this fatty acid is crucial for their survival. Some researchers suggest that lipids serve as energy source for displacement and for the production of ectoplasmatic nets. Although DHA is preferentially used as energy source under starving conditions, the PUFAs could also protect cells from oxidative stress when nutrients are depleted, because of their antioxidant properties. In addition, cells with higher PUFA contents are gravitationally less dense than those containing more saturated fatty acids, a difference that could induce displacement to nutrient-rich environments (Jain *et al*., 2007).

Because of the industrial and commercial interest originated from recent discoveries of physiological DHA importance in human beings, animals and fish, thraustochytrids are currently objects of study, and characteristics of new strains are periodically reported. At present, two isolates, *Schizochytrium* sp. 20888 and *Ulkenia* sp., are being exploited for commercial DHA production (Barclay *et al*., 2005; Kiy *et al*., 2005).

DHA content in thraustochytrid cells is strongly dependent on environmental

factors such as temperature, salinity, and culture medium composition. Thraustochytrids are able to grow on different organic C sources and both organic and inorganic N sources (Goldstein, 1973); nevertheless, the composition of growth medium also influences biomass production and lipid composition, thus DHA yield is also affected. This work reviews the effects of C source on DHA production by different thraustochytrid strains. Results obtained by different investigators on the use of alternative and low cost nutrient sources for DHA production by thraustochytrids are also presented. Finally, results obtained in the production of DHA by thraustochytrids under different cultivation strategies were described.

Effect of medium composition on DHA production by different thraustochytrid strains

For the cultivation of different thraustochytrid strains, glucose is generally the first C source evaluated. On the other hand, organic (yeast extract, tryptone, polypeptone) and inorganic (ammonium sulfate, ammonium chloride) N sources have been tested at different concentrations, because the C/N ratio influences lipid accumulation in some strains. Lipid accumulation, in oleaginous microorganisms, occurs when the medium contains a C source excess and a limiting N amount. Thus, when the organism grows, it quickly exhausts N source but it continues assimilating the C source that is channeled directly into lipid synthesis (Ratledge and Wynn, 2002). However, cultural conditions involving low N concentrations decrease cell growth, hence, lower lipid and DHA yields are obtained.

One of the first strains evaluated for the production of DHA was *T. aureum* ATCC 34304. Lipid content in the biomass of this microorganism was dramatically influenced by the medium composition (concentrations of glucose, organic N source, vitamins and salts) (Bajpai *et al*., 1991). An increase of glucose concentration from 5 to 20 g L^{-1} increased both the percentage of lipids in the biomass (from 2.7 to 16.5%) and DHA yield (from 26 to 270 mg L^{-1}) in a 6-day incubation.

Growth and DHA production by *T. roseum* ATCC28210 in media containing starch (25 g L^{-1}) as C source was studied by Singh and Ward (1996); ammonium sulfate (0.2 g L^{-1}), and sodium glutamate (2 g L^{-1}) were used as N sources. Biomass and DHA yields of 6.1 g L^{-1} and 528 mg L^{-1}, respectively, were obtained after 5 days. Growth medium supplemented with yeast extract (2 g L^{-1}) allowed to increase biomass and DHA yields to 8.6 g of dry cell weight (DCW) per L and 892 mg L^{-1}, respectively. The increase of KH_2PO_4 concentration from 0.1 g L^{-1} to 0.2 g L^{-1} improved DHA yield by 40%, whereas a reduction in NaCl concentration (from 25 g L^{-1} to 10 g L^{-1}) had no effect on DHA yield. Attempts to increase biomass and DHA yields were made by supplying additional doses of carbohydrate equivalent to 10 g L^{-1} after both 4 and 6 days in a fed-batch system. This cultivation condition increased dramatically biomass yield, and the contents of lipid and DHA in the biomass. Maximum biomass (12.1 g L^{-1}), lipid in biomass (23.7%), and DHA in biomass (118 mg g^{-1}) were obtained after 8 days; final DHA yield was 1433 mg L^{-1}. The same process carried out supplying glucose instead of starch resulted in a lower DHA yield (1130 mg L^{-1}). In a fed-batch fermentation carried out for 12 days adding aliquots containing starch (10 g L^{-1}), sodium glutamate (0.8 g L^{-1}), ammonium sulfate (0.08 g L^{-1}), KH_2PO_4 (0.08 g L^{-1}) and $MgSO_4$ (2 g L^{-1}) maximum biomass and DHA yields were

17.1 g L^{-1} and 2 g L^{-1}, respectively. These results indicate that biomass growth and DHA production can be increased by feeding not only the C source but also the N sources and salts.

Conditions for DHA production at a high level by *Schizochytrium* sp. SR21 through changes in the composition of the growth medium were reported by Yaguchi *et al*. (1997). With medium I [glucose 60 g L^{-1}, corn steep liquor 0.7 g L^{-1}, ammonium sulfate 2 g L^{-1}, KH_2PO_4 3 g L^{-1}, in 50% artificial sea water (ASW)] strain SR21 produced 21.9 g DCW L^{-1} and 5.1 g DHA L^{-1} at 65 h. By increasing twice C and N amounts, higher biomass (48.1 g L^{-1}) and DHA (13.3 g DHA L^{-1}) yields were obtained at 96 h. It was possible to produce 59.2 g DCW L^{-1} and 15.5 g DHA L^{-1} in the fermentation of a medium that contained glucose 150 g L^{-1}, corn steep liquor 1.75 g L^{-1}, ammonium sulfate 5 g L^{-1}. Lipid content in the cells of strain SR21 increased with cell growth. In the early growth phase, neutral and polar lipids were produced in about equal amounts; however after this phase the content of neutral lipids increased with cell growth. In the stationary growth phase lipid profile became 95% neutral lipids and 5% polar lipids. Neutral lipids were composed of 35% DHA and 6% DPA while the content of these fatty acids in polar lipids was 63.5% and 24.7%, respectively.

The biomass, lipid and DHA production by strain G13, a thraustochytrid strain resembling *Schizochytrium mangrovei*, was evaluated in a medium that contained glucose (40 g L^{-1}), yeast extract (20 g L^{-1}), and ASW (27 g L^{-1}) (Bowles *et al*., 1999). Maximum biomass yield (14 g L^{-1}) was reached at 41 h, close to the point of glucose exhaustion. This was also the point at which the lipid content in the biomass reached a maximum of 56% (w/w). The DHA content in the lipids remained stable at around 28% (w/w) from 63 to 120 h cultivation. Composition of the medium was modified further by substituting the ASW with sodium sulphate (20 g L^{-1}) as source of sodium, and decreasing the concentration of yeast extract (from 20 to 5 g L^{-1}). In this modified growth medium glucose was exhausted at 24 h, point at which biomass yield was 14 g L^{-1}. At 41 h the content of lipids in the cells was maximal (78% w/w) and the percentage of DHA in the lipids was 18%. A prolonged incubation allowed to increase DHA yield; maximum DHA yield equal to 2.17 g L^{-1} was achieved at 107 h.

A culture medium contained glucose (30 g L^{-1}), yeast extract (5 g L^{-1}), and polypeptone (15 g L^{-1}) in ASW (50%) was used for the cultivation of *Schizochytrium limacinum* KH105, strain that in addition to produce DHA is also able to synthesize carotenoids (Aki *et al*., 2003). A semi-optimized growth condition, in a baffled flask, yielded 11.5 g DCW L^{-1}, 5.3 g of total fatty acids (TFA) per L, and 1.2 g DHA L^{-1} after 2 days. In addition to glucose, strain KH105 was also able to assimilate D-fructose, glycerol, and L-arabinose. This assimilation profile is distinct from those of other *Schizochytrium* strains; for instance, *S. aggregatum* ATCC 28209 can propagate in a medium containing maltose or cellobiose, but not D-fructose as a sole C source (Bahnweg 1979).

Schizochytrium sp. F26-b, a strain isolated from Ishigaki island in Japan, produced 3.5 g CDW L^{-1} and 915 mg of lipids L^{-1} when it was inoculated into a medium containing glucose (3%), yeast extract (1%) in 50% ASW, pH 6.0 (Abe *et al*., 2006). The fractions of neutral lipids, glycolipids, and phospholipids corresponded to 66%, 7%, and 22%, respectively. The major fatty acids in the total lipid fraction were pentadecanoic acid (C15:0) and DHA; together they

represented more than 67.1% of all the fatty acids. The highest proportion of DHA was found in the phospholipid fraction, in which DHA was about 50% of the fatty acids.

Schizochytrium sp. ONC-T18, isolated from mangrove leaves collected at Advocate Harbour, Nova Scotia, was selected by Burja *et al*. (2006) for culture optimization. This strain has a high biomass production rate, 25% higher than that exhibited by *Schizochytrium* sp. ATCC 20891; moreover this strains is able to accumulate up to 80% of its biomass as lipid when it was cultivated in a media containing glucose (60 g L^{-1}), yeast extract (2 g L^{-1}) and monosodium glutamate (8 g L^{-1}) with sea salts at 6 g L^{-1}. Glucose, DL-malic acid, D-fructose, D-xylose, fumaric acid, D-cellobiose, pyruvic acid, α-D-lactose, 5-keto-D-gluconic acid, and glycerol supported good cell growth and DHA yield. A high content of DHA (more than 20%) in the biomass was obtained with these C sources. In contrast, di- and polysaccharides gave poor cell growth.

Alternative sources of nutrients for the production of DHA by thraustochytrids

A favorite substrate for thraustochytrids is *Pinus* pollen that is often used for their isolation. The pollen wall is impregnated with sporopollenin, a polymeric material composed of oxidized carotenoids and carotenoid esters, which is extremely resistant to acid and alkali attack. It has been suggested that the ability of thraustochytrids to break down sporopollenin walls together with chitin and cellulose could be due to a long evolutionary history or a very specialized adaptation (Chamberlain and Moss, 1988).

Various kinds of alternative C and organic N sources were used to investigate the cell growth and DHA yield of *S. limacinum* SR21 (Yokochi *et al*., 1998). Compared with glucose, oleic acid and linseed oil gave similar yields of biomass (more than 12 g L^{-1}) and total fatty acids (more than 2.5 g L^{-1}). However, the DHA content in the biomass grown in these C sources was lower than 10%. Best N source for the production of fatty acids was corn steep liquor; the ratio of total fatty acid content to biomass with corn steep liquor was twice as much as that obtained with yeast extract, the most used N source for the cultivation of thraustochytrids. Little differences in fatty acid composition were found when corn steep liquor was used instead of yeast extract as N source; however, the highest DHA yield (1.7 g L^{-1}) was obtained with corn steep liquor. The higher accumulation of lipids in the biomass grown with corn steep liquor compared with that obtained with yeast extract was attributed to the lower nitrogen level (5.5%) in corn steep liquor compared with the one found in yeast extract (10%). Thus, the more N limiting condition provided by the growth medium that contained corn steep liquor would explain the higher lipid accumulation.

A residue from soymilk production denominated okara was evaluated as nutrient source for the growth of different thraustochytrid strains by Fan *et al*. (2001). Okara is nutritionally rich, with 52% carbohydrate, 27% protein and 12% fats (Ma *et al*., 1997). The okara medium was composed of 10 g of pulverized okara and 1 L of 15% ASW at pH 6. DHA yield in the okara medium was much lower (35.3-72.1 mg L^{-1}) when compared with the yields obtained in a glucose-yeast extract medium (747.7-2778.9 mg L^{-1}). Composition of the growth medium affected significantly the fatty acid profile of the mangrove thraustochytrid strains; while main fatty acids in cells grown in the glucose medium were palmitic acid and DHA, the cells grown in the okara

medium contained more oleic and linoleic acids. The authors attributed the observed differences to the fact that simple sugars, such as glucose, are more readily utilizable than the recalcitrant polymers, such as the cellulose, available in okara for bioconversion by thraustochytrids.

Wastewater from barley shochu distillery as nutrient source for the growth of *Schizochytrium* sp. KH105 was evaluated by Yamasaki *et al*., (2006). The culture medium was prepared by adding various amounts of glucose and artificial sea salts in wastewater followed by pH adjustment. Due to the poor growth observed in the absence of glucose, the effect of wastewater concentration on the lipid production was examined in the medium containing glucose (80 g L^{-1}) and 50% shochu distillery wastewater at pH 7.5. Maximum of DHA yield obtained after 4 days was 3.4 g L^{-1} (25.8% of the TFA; 115 mg of DHA per g of cells), which was almost equivalent to the yield obtained in a more expensive culture medium containing polypeptone and yeast extract (Aki *et al*., 2003).

Crude glycerol, from biodiesel production, was used as C source for the cultivation of *S. limacinum* SR21 (Chi *et al*., 2007). Crude glycerol used by these investigators contained 83% (w/w) glycerol and 12% (w/w) methanol. The used N sources were tryptone (1 g L^{-1}), and yeast extract (1 g L^{-1}). Results were compared with those obtained with glucose and pure glycerol. DHA yield and DHA productivity showed similar trends as those exhibited by the CDW. In the fermentations of glucose, pure glycerol and crude glycerol CDW yields were 18.47 g L^{-1}, 14.43 g L^{-1}, and 18.04 g L^{-1}, respectively. The effects of crude glycerol concentration on cell growth and DHA production were investigated by including crude glycerol at levels ranging from 75 to 150 g L^{-1} into ASW; growth was inhibited when the crude glycerol concentration exceeded 100 g L^{-1}. Under the optimum culture condition (100 g L^{-1} crude glycerol, 19.2°C and 1.0 g L^{-1} ammonium acetate), DHA content in the biomass was more than 20% and DHA yield was 4.91 g L^{-1}.

Schizochytrium mangrovei sp. Sk-02 isolated from samples collected in a mangrove forest was used to evaluate the effect of coconut water (CW) in a glucose-yeast extract diluted in sea water medium on DHA yield (Unagul *et al*., 2007). CW contained a significant amount of monosaccharides in the form of glucose and fructose (total 11 g L^{-1}), and sucrose (6.7 g L^{-1}); together these sugars accounted for almost half of the dry solids in CW while N was a minor component. Optimal CW-level was 33% (v/v), resulting in a biomass yield of 28 g L^{-1} with a DHA content in the biomass of 20% (w/w). DHA yield was 6 g L^{-1}, almost 50% higher than the yield obtained in the non-supplemented cultures at the same initial sugar concentration.

Soybean cake hydrolysate was evaluated as a cheap N source for the production of DHA by *S. limacinum* OUC88, strain derived from *S. limacinum* SR21 by UV-mutagenesis (Zhu *et al*., 2008). Different C sources (glucose, fructose, soluble starch, potato powder and glycerol) were used separately at a concentration of 30 g L^{-1} in the basal medium that contained soybean cake hydrolysate (20 g L^{-1}) and 50% natural seawater. Among the C sources tested, potato powder yielded the highest biomass concentration (14.05 g DCW L^{-1}) while maximum DHA yield (1.78 g L^{-1}) was achieved in the glucose containing medium in the presence of soybean cake hydrolysate. This DHA yield was also almost twice as much that obtained with yeast extract. In a further optimization the highest biomass yield (25.92 g CDW L^{-1}) was observed in the medium containing 90 g L^{-1} of glucose and 60 g L^{-1} soybean

cake hydrolysate. However, the maximum DHA yield (4.08 g L^{-1}) was obtained in the medium containing 60 g L^{-1} of glucose and 20 g L^{-1} soybean cake hydrolysate.

Liquid residues from beer (RB) and potato (RP) processing were used as C sources for the production of DHA by Thraustochytriidae sp. M12-X1 (similar to *Thraustochytrium kinnei*, 99% identity) and Thraustochytriidae sp. C41 (similar to *Schyzochytrium minutum*, 97% identity) in shaking flasks (Quilodrán *et al*., 2009). For both strains, RB supplemented with yeast extract and monosodium glutamate, at 2 g L^{-1}, permitted the production of the highest biomass concentration (2.3 g L^{-1}). In both strains, the relative content of the different fatty acids was significantly affected by the carbon source; fatty acids from cells grown in the media that yielded more biomass contained more palmitic acid. On the contrary, fatty acids from cells grown under limiting conditions contained more DHA. Fermentation of RB medium that contained yeast extract and monosodium glutamate by Thraustochytriidae sp. M12-X1 showed the highest DHA productivity (55.1 mg $L^{-1}day^{-1}$).

The same residues were used as nutrient sources for cultivating a native *Ulkenia* strain, able to produce DHA and astaxanthin (Quilodrán *et al*., 2010). RB, as the only source of nutrients, supported cell growth (8.1 g DCW L^{-1}) and DHA production (576 mg L^{-1}); however, its supplementing with yeast extract, monosodium glutamate and B-vitamins (thiamine, biotin and cobalamin) dramatically increased DHA productivity [540 mg $L^{-1}day^{-1}$] and the yields of biomass (14.9 g L^{-1}) and DHA (2698 mg L^{-1}).

Cultivation strategies for the production of DHA by thraustochytrids

Ganuza *et al*. (2008) evaluated the capacity of the pH-auxostat system to optimize growth of *Schizochytrium* sp. G13/2S, strain derived by nutritional selection from strain G13. The medium contained glucose and ammonia at concentrations of 150 g L^{-1} and 2.4 g L^{-1}, respectively. pH-auxostat fermentation was carried out controlling pH at 7 with NH_4OH; by this strategy ammonia concentration was maintained between 300 mg L^{-1} and 400 mg L^{-1}. As the initial growth of the cultures was not restricted by nutrient limitation, the cells did not enter to a lipid-accumulation phase during the course of the fermentation run. Thus, *Schizochytrium* sp. G13/2S was grown under non N limiting condition to a high biomass density (60 g L^{-1}) in 2 days. The content of total fatty acids in the cells remained constant at about 25% (w/w) in which DHA was found near 40% of the total fatty acids throughout growth; thus DHA and total fatty acids were synthesized entirely growth-associated.

Lipid accumulation in *Schizochytrium* G13/2S cultured in batch and continuous systems under different C or N restrictions and at different dilution rates was studied by Ganuza *et al*. (2007). Lipid production in batch cultivation of *Schizochytrium* G13/2S with glucose (40 g L^{-1}) and glutamate (2 g L^{-1}) increased markedly during the late growth phase, reaching about 35% of DCW. This value was kept constant for the rest of the culture until residual glucose was completely depleted. At the stationary phase, the percentage of DHA in the total fatty acids was between 43-47%.

Chemostat culture of *Schizochytrium* G13/2S was conducted using an N limited medium (glutamate 2 g L^{-1} and glucose 40 g L^{-1}) under dilution rates ranging from 0.08 to 0.02 h^{-1}. DCW and the content of total fatty acids increased linearly as dilution rate decreased. The highest biomass (7.7- 6.2 g DCW L^{-1}) and lipid accumulation (31–28% TFA) were obtained at the lowest assayed dilution rates (0.02 and 0.04 h^{-1}, respectively).

CONCLUSIONS

Thraustochytrids as microorganisms for biotechnological production of DHA are current objects of study. At present, most studies focus on the screening for better DHA producer strains. For each new strain, fermentation studies for defining growth medium composition and cultivation techniques need to be carried out in order to improve DHA yield. Although conditions for the production of biomass yields as high as 60 g L^{-1} and DHA yields equal to 15.5 g L^{-1} have been reported, these values could be considered as extraordinary exceptions. As the microbial oil production has to be profitable attempts to reduce production costs are currently tested by different investigators. In this sense, low cost nutrient sources, such as wastewater from the food industry, should be identified. On the other hand, few works present results other than those obtained in flask experiments. The scale-up to higher production levels could offer several challenges due to the high biomass concentration needed in order to increase DHA productivity.

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