Advance Journal of Food Science and Technology 4(5): 257-264, 2012
ISSN: 2042-4868 E-ISSN: 2042-4876
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Submitted: July 17, 2012 Accepted: August 17, 2012 Published: October 20, 2012

Effect of the Concentration of Glucose in the Docosahexaenoic Acid (DHA) Production by *Thraustochytrium* sp., ATCC 26185

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Abstract: Intake of adequate levels of ω-3 and ω-6 fatty acids plays an important role in the prevention and modulation of various diseases. Several parameters, including the carbon source in the culture of microorganisms have been reported to be essential in the biosynthesis and accumulation of Polyunsaturated Fatty Acids (PUFAs). This work aimed to study the effect of different concentrations of glucose for the production of PUFAs, especially Docosahexaenoic Acid (DHA), from *Thraustochytrium* sp. ATCC 26185. The following concentrations of glucose were studied: 30 g/L, 60 g/L and 0.10 g/L.h (fed-batch). The contents of biomass, glucose consumption, total nitrogen and the production of PUFAs were evaluated. The highest content of biomass (30.2 g/L) was observed using 30 g/L glucose as carbon source. The majority composition of PUFAs in *Thraustochytrium* sp. ATCC 26185 was DPA (21-24%) and DHA (68-71%), regardless of type and time of culture. The experiment that used 30 g/L glucose for 120 h of culture showed the highest DHA yield (1.16 g/L), demonstrating that the growth of *Thraustochytrium* sp. ATCC 26185 and accumulation of PUFAs, particularly DHA is dependent on the concentration of the available carbon source for its consumption as well as the growing period.

Keywords: Carbon source, docosahexaenoic acid, polyunsaturated fatty acid, *Thraustochytrium* sp.

INTRODUCTION

In recent years, a growing demand for functional foods has been observed, both to increase the quality of life and to aid the treatment of nutritional deficiencies.

There is increasing for Polyunsaturated Fatty Acids (PUFAs) due to beneficial effects on human health, which range from prevention of cancer and cardiovascular diseases to treatment in mental illness (Bergé and Barnathan, 2005). Furthermore, PUFAs are among the nutrients of interest, mainly for carrying important physiological functions, because they are components of cell membranes in brain cells (Kang and Leaf, 1996; Sijtsma and Swaaf, 2004; Wall *et al*., 2010).

Among the PUFAs, we can highlight the Docosahexaenoic Acid (DHA, C22:6 ω-3) and Docosapentaenoic Acid (DPA, C22: 5 ω-6).

Studies show that intake of DHA develops the brain of newborn children, helping with the increase of intelligence, verbal skills and reasoning (Shwu-Tzy *et al*., 2005). For this reason, this fatty acid has been incorporated in fortifying infant formulations in various parts of the world. Additionally, the DHA is important in the treatment of atherosclerosis, rheumatoid arthritis, Alzheimer's disease (Simopoulos *et al*., 1991; Schaefer *et al*., 2006; Corsinovi *et al*., 2011) as well as in the prevention of breast and colon cancer. Just as DHA, DPA is important for human health, as it prevents the occurrence of various diseases such as cardiovascular accidents (heart attack, thrombosis, atherosclerosis), diabetes, inflammatory and antirheumatic processes (arthritis, osteoporosis, asthma) (Rose and Connolly, 1999; Covington, 2004; Raghukumar, 2008; Seminario, 2011).

Currently the main commercial sources of these compounds, especially of DHA are oils from marine fish. However, their widespread use is limited due to seasonal variations in fish, marine pollution and the high cost of the process of getting this oil. Studies show that in less than 10 years, the production of PUFAs from current sources will be unsustainable for the desired expansion of the market (Sijtsma and Swaaf, 2004).

For these reasons, there is an intense search for alternative sources of oils rich in PUFAs, motivated by the high cost and environmental concern due to reduced stocks of fish used for human and animal food.

Several groups of microorganisms have the ability to synthesize large quantities of bioactive compounds

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such as DHA, among which stands out the genus *Thraustochytrium*, which can produce high levels of DHA and can reach 0.51 g/L, corresponding to 51% (w/w) of the total lipids present in cell biomass (Bajpai *et al*., 1991a, b). In the research of Burja *et al*. (2007), *Thraustochytrium* sp. ONC-T18 produces high amounts of DHA, corresponding to 23.5% of total lipids.

According to Gupta *et al*. (2012), there has always been confusion among researchers regarding the taxonomical classification of Thraustochytrids. The taxonomical structures have been first established and then abolished quite often in the process of developing taxonomy for Thraustochytrids, which has added to ambiguity regarding its structural and functional behaviour. Thraustochytrids are large-celled marine heterokonts and classified as oleaginous microorganisms due to their production of ω-3-fatty acids.

With the development of DNA sequencing methods and electron microscopic studies of ultrastructure, Thraustochytrids were subsequently designated as a unique group. The genera included in this group are *Thraustochytrium*, *Schizochytrium*, *Japonochytrium*, *Aplanochytrium*, *Elina*, *Labyrinthula* (or *Labyrinthuloides* or *Labyrinthulomyxa*). *Thraustochytrium* has been included in Thraustochytriaceae family. Hence, after numerous revisions, Thraustochytrids proved to be a distinctive and characteristic division of protists in which the members can be classified under the Thraustochytriales order (Metz *et al*., 2010).

Since they are heterotrophic microorganisms, there is no power generation by photosynthesis, so there is the need to supply power through of carbon sources. For this purpose, glucose was used as the best source of carbon in growing organisms from the family Thraustochytrids (*Thraustochytrium*) for biomass and PUFAs production (Singh *et al*., 1996a; Yokochi *et al*., 1998; Raghukumar, 2008).

The growth of Thraustochytrids and their fatty acid composition depend on the nutrients and growing conditions (Chihib *et al*., 2005). However, the choice of carbon source is not the unique variable to consider for the production of biomass and PUFAs, but is important to test the more suitable glucose concentration for these microorganisms. Glucose is the commonly used and economical substrate for microbial lipid production (Singh *et al*., 1996a; Shene *et al*., 2010).

Thus, the aim of this work was to study the effect of glucose concentrations in the culture of the microorganism *Thraustochytrium* sp., ATCC 26185 for production of PUFAs, particularly DHA.

MATERIALS AND METHODS

Microorganism: The *Thraustochytrium* sp., ATCC 26185 strain used in this study was obtained from American Type Culture Collection (Manassas, VA, USA).

Preparation of inoculum: Cells from the microorganism *Thraustochytrium* sp., ATCC 26185 stored at 4°C in potato dextrose agar were transferred to 500 mL flasks containing 100 mL medium composed (g/L) of: yeast extract (1.0), peptone (1.0) and glucose (5.0) in seawater (1.5% w/v). The glucose was sterilized separately. Cells were incubated in an orbital shaker (IKA, 260B KS) at 30°C, 150 rpm, without light, for 48 h.

Culture conditions: The culture was carried out in a bench bioreactor (Sartorius Stedim Biotech®, Biostat BPlus, equipped with pressure flow meters and controllers of gases and liquids) and the medium was composed of (g/L): KH_2PO_4 (1.54), $(NH_4)_2SO_4$ (6.25), $MgSO_4.7H_20$ (2.62), NaCl (0.71), yeast extract (8.8) and different concentrations of glucose (30 g/L, 60 g/L and 0.10 g/L.h fed-batch-previous assays) as carbon source. All components were dissolved in 3.15 L of seawater (1.2% w/v). The sterilization of yeast extract and glucose were performed individually at 121°C for 15 min in an autoclave (Cetorclav, CV-EL-18 L). The bioreactor was sterilized by autoclave (AJC, Uniclave 77-127 L) for 60 min and the other medium components were sterilized by membrane filtration (0.22 μm, Millipore). The dissolved components (sterilized) were added to the bioreactor together with metal solutions (mg/L), $MnCl_2 \cdot 4H_2O$ (3.0), $ZnSO_4.7H_2O$ (3.0), $CoCl_2 \cdot 6H_2O$ (0.04), $Na_2MoO_4 \cdot 2H_2O$ (0.04), $CuSO_4 \cdot 5H_2O$ (2.0), $NiSO_4 \cdot 6H_2O$ (2.0), $FeSO_4.7H_2O$ (10.0) and vitamin solutions (mg/L), thiamine (9.5) and calcium pantothenate (3.2), previously sterilized by membrane filtration (0.22 μm, Millipore). Finally 350 mL of inoculum (10% v/v relative to the total volume of culture medium) was added. The experiments were conducted at 23°C, shaking at 100 rpm and pH 6.0, adjusted with NaOH (4 M). Within 96 h of culture the concentrations of dissolved oxygen in the medium were maintained at 5%, controlled by aeration (0-2.5 vvm), followed by injection of 0-0.25 vvm of pure oxygen. After this period, the injection of air and oxygen were discontinued.

Determination of the biomass content: The cell concentration was determined at 24 h intervals, filtering an aliquot of the previously weighed culture medium in glass microfiber (GF/C: 1.2 μm, Whatman) according to Min *et al*. (2012) with some modifications. Biomass in microfiber was washed twice with distilled water,

dried at 60°C in an oven (Memmht) for 24 h. The biomass content was determined by the difference between the weight of glass microfiber containing biomass dry weight and the microfiber glass without the biomass, prior to filtration.

Determination of glucose uptake: Sugars were measured in the culture supernatant at 24 h intervals by spectrophotometric method proposed by Miller (1959) using UV/VIS dual beam absorption spectrophotometer (ATI UNICAM Helios, Alpha, UK).

Determination of total nitrogen: To quantify the total nitrogen content (N: organic, nitrite, nitrate and ammonia), 10 mL of culture supernatant, collected every 24 h, were transferred to macro Kjeldahl tubes and 10 mL of H_2SO_4 (36 N) and Kjeltabs S/3.5 catalyst (3.5 g K_2SO_4 and 3.5 mg Se) (Foss Analytical) were added. The sample was then subjected to mineralization in a block digester (Tecator, Digestion System 20-1015) at maximum temperature of 400°C until it became colorless. The sample was cooled to room temperature and added with 1 g of league Devarda (Sigma-Aldrich) and 50 mL of NaOH (35%). Steam distillation was carried out (Velp Scientifica, 152 UDK) for 15 min, the condensate was collected in 25 mL of H_3BO_3 (4%)* and titrated with HCl (0.1 N).

*H_3BO_3 (4%): 40 g/L of H_3BO_3 + 10 mL of bromocresol green (0.1 g of bromocresol green in 100 mL of ethanol) + 6.66 mL of methyl red indicator (0.1 g of methyl red in 100 mL of ethanol). The concentration of total nitrogen was expressed according to the Eq. (1):

$$\text{Total Nitrogen (g/L)} = \{[\text{Volume (mL) HCl}_{\text{sample}} - \text{Volume (mL) HCl}_{\text{white}}] \times 1.4\}/10 \quad (1)$$

Determination of fatty acids profile: Samples of the culture collected at intervals of 24 h, were centrifuged (Kubota, 6800) at 8742 g for 15 min at 4°C and the biomass washed with distilled water and centrifuged again. This process was repeated twice. The biomass was frozen at -20°C and dry for 48 h in a lyophilizer (Heto, Power Dry LL 3000). Lyophilized cell biomass between 20 and 100 mg was weighed and added to 50 µL of internal standard solution C23:0 (50 mg/mL) in order to express the results in g of fatty acid/g of biomass lyophilized. The methyl esters of fatty acids were prepared by esterification by acid catalysis using the method of Lepage and Roy (1986) modified by Cohen *et al*. (1988), analyzed by gas chromatograph Varian 3800 CP (Walnut Creek, CA, USA) equipped with autosampler, injector and flame ionization detector (FID), both at 250°C. The separation occurred using a polyethylene glycol capillary column DB-WAX (30 m length, 0.25 mm internal diameter and 0.25 µm thick) Agilent (Albertville, MN, USA) heated at 180°C (5 min) gradually increasing every 4°C/min up to 220°C (holding for 25 min) and in increase gradually (20°C/min) to 240°C (holding for 15 min). The methyl esters were identified in the sample by comparison with the retention times of chromatographic patterns Sigma-Aldrich Co. (St. Louis, MO, USA).

The data were subjected to Analysis of Variance (ANOVA) and significant differences were identified by test of comparison among the means at 5% significance level. Before performing ANOVA was necessary to check whether the data were normal and their variances were seen to be equal (Triola, 1999).

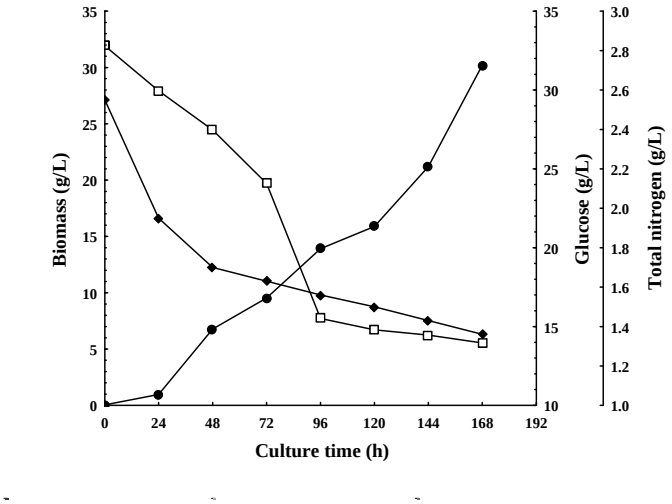
The study was carried out in 2011 in the Portuguese Institute of Sea and Fisheries Research I.P.-INRB/IPIMAR on Lisbon, Portugal.

RESULTS AND DISCUSSION

Determination of biomass content, glucose consumption and total nitrogen: Figure 1, 2 and 3 show the average concentrations of the contents of cell biomass, glucose and total nitrogen, for the culture of *Thraustochytrium* sp., ATCC 26185. In the culture which used glucose concentration 30 g/L as carbon source (Fig. 1), it can be observed that the maximum concentration of biomass (30.2 g/L) was reached after 168 h of culture, with average productivity 0.18 g/L.h biomass.

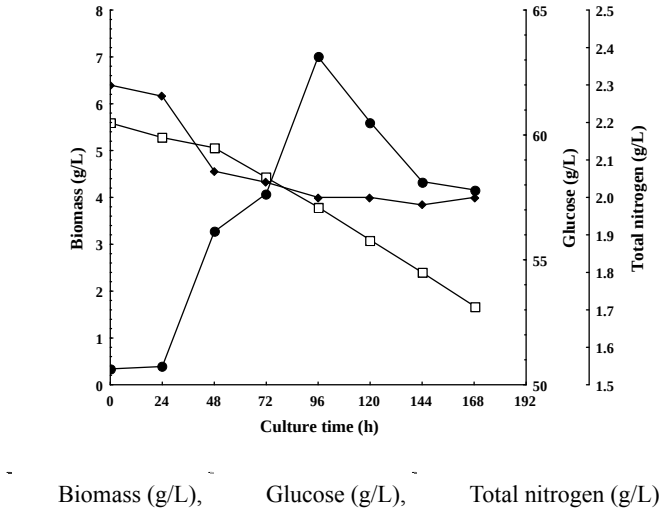
The average consumption of glucose in this experiment was 0.11 g/L.h and the highest specific rate of consumption of substrate (0.13/h) was within 24 h of culture. This experiment showed also that for each gram of glucose consumed 1.6 g of biomass were produced ($Y_{Biomass/Glucose}$: 1.6). For the nitrogen provided, the maximum specific consumption speed total (0.03/h) was in the first 24 h, with an average consumption of 0.007 g/L.h, presenting a substrate conversion factor in product of 25.3 ($Y_{Biomass/Nitrogen}$). From Fig. 2 it can be seen that the higher concentration of biomass (7.0 g/L) was achieved after 96 h of culture, using 60 g/L glucose as carbon source, with a average productivity 0.07 g/L.h of biomass. Maximum specific glucose consumption speed (0.05/h) was at 24 h, with an average consumption of 0.04 g/L.h This culture had a conversion of glucose to product ($Y_{Biomass/glucose}$) of 1.97.

Maximum specific consumption speed of total nitrogen (0.03/h) was in 24 h of culture and the average consumption of this substrate was 0.003 g/L.h. Each gram of nitrogen intake was converted to 22.2 g of biomass ($Y_{Biomass/nitrogen}$). Providing glucose as carbon source under fed-batch system (0.10 g/L.h glucose), it was found that the higher biomass concentration



Biomass (g/L), Glucose (g/L), Total nitrogen (g/L)

Fig. 1: Concentrations of biomass, glucose and total nitrogen during the culture *Thraustochytrium* sp., ATCC 26185 using 30 g/L glucose as carbon source



Biomass (g/L), Glucose (g/L), Total nitrogen (g/L)

Fig. 2: Concentrations of biomass, glucose and total nitrogen during the culture *Thraustochytrium* sp., ATCC 26185 using 60 g/L glucose as carbon source

(13.0 g/L) was reached the 96 h (Fig. 3), with a maximum productivity 0.13 g/L.h biomass. At the end of 48 hours the highest specific glucose consumption speed (0.02/h) occurred with an average consumption of 0.10 g/L.h and conversion factor (1.3) of glucose to product ($Y_{Biomass/Glucose}$). Maximum specific consumption speed of total nitrogen (0.03/h) was within 24 h of culture, the average consumption of this substrate was 0.008 g/L.h The conversion factor ($Y_{Biomassa/Nitrogen}$: 15.3), indicated that each gram of nitrogen consumed may produce 15.3 g of biomass.

Therefore, the culture which employed the glucose concentration of 60 g/L was the one which consumed smaller quantities of substrate (0.04 g/L.h glucose and 0.003 g/L.h total nitrogen) thus formed a smaller amount of biomass (0.07 g/L.h). This may have been due to high concentration of glucose (60 g/L) provided at the beginning of the experiment, which eventually inhibits the microorganism, probably due the higher C/N ratio. Between the three studied glucose concentrations the experiment using 60 g/L showed the higher C/N ratio. At a higher C/N ratio, synthesis of nitrogen containing compounds such as protein and nucleic acids is curtailed and the growth is inhibited (Roessler, 1990) or more slowly (Burja *et al*., 2006).

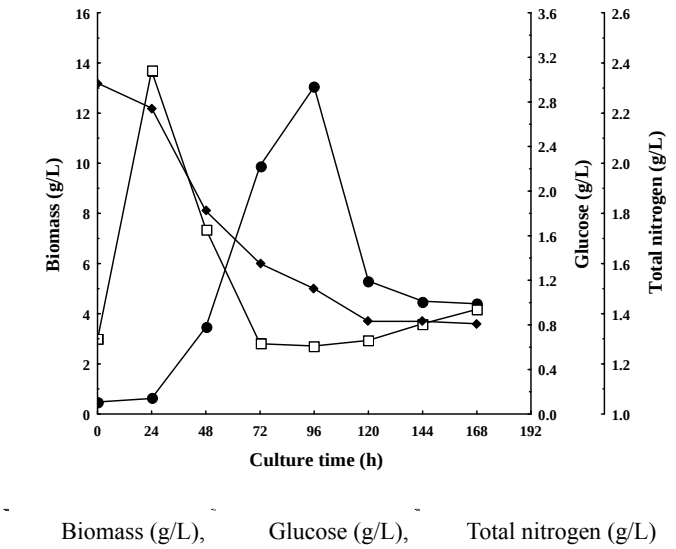


Fig. 3: Concentrations of biomass, glucose and total nitrogen during the culture *Thraustochytrium* sp., ATCC 26185 using 0.10 g/L.h (fed-batch) glucose as carbon source

This inhibition was also observed in studies conducted by Ka (2004), when a high concentration of glucose in the culture of *Thraustochytrium* sp., 26185 was used. Furthermore, results obtained for *Thraustochytrium aureum*, with glucose concentrations above 10 g/L were inhibitory (Iida *et al*. 1996).

The fed-batch culture system consumed approximately the same amounts of substrate (0.10 g/L.h glucose and 0.008 g/L.h nitrogen) as the culture which used 30 g/L glucose (0.11 g/L.h of glucose and 0.007 g/L.h nitrogen). However, their efficiency in the conversion to product (0.13 g/L.h) was not observed as in the experiment which used 30 g/L glucose (0.18 g/L.h). This could be due to the effect that under culture conditions, had not been the formation of biomass as in the culture that used 30 g/L glucose concentration, but the metabolism of other components in larger quantities such as ATP, which are used in performing various physiological reactions (absorption, excretion, etc.) and biosynthesis, necessary to maintain the organisms.

In the study by Byung-Ki *et al*. (2002) the maximum biomass concentration reached (4.5 g/L) was achieved after 168 h of culture of the *Thraustochytrium aureum* ATCC 34304 using an initial concentration of glucose of 29 g/L, with a conversion of sugar in biomass of 0.53 ($Y_{Biomass/Glucose}$). These authors found also that the conversion of sugar into biomass decreases with increasing initial concentration of glucose supplied to the culture, which was demonstrated also in this study.

Determination of fatty acids profile: Figure 4 shows the average values of the content of PUFAs related with the culture time of *Thraustochytrium* sp., ATCC 26185.

Through statistical analysis confirmed the normality of results by the Kolmogorov-Smirnov test and homocedasticity by Cochran test. From the results PUFAs it is possible to apply ANOVA, followed by the means comparison test (Tukey) at the 5% significance level, from which we can be concluded that there was significant differences between the culture times in all glucose concentrations studied, except between 96 and 144 h for culture that used 30 g/L glucose and between 0 and 24 h and 144 and 168 for the assays under fed-batch regime.

The increased production of PUFAs (1.68 g/L) was observed in the experiment that used 30 g/L glucose after 120 h of culture. In the culture with 60 g/L glucose, the greatest amount of PUFAs (0.28 g/L) was obtained at 72 h, as well the maximum production of PUFAs (0.60 g/L) in the culture under fed-batch regime.

The Fig. 5 shows the fatty acids profile for each experiment in the times that were achieved the higher PUFAs concentrations. The high saturated fatty acid content were in C15:0 (27-35%) and C16:0 (31%). In the present study, DHA was the predominant PUFA ranging from 26 to 38.5% of the total fatty acids (Fig. 5a). Similar trends were observed in the study of Weete *et al*. (1997), where the DHA yield was 25 to 32% total fatty acids using the same strain. DHA and DPA were the major PUFAS detected (Fig. 5b). This result is in agreement with that of Taoka *et al*. (2011), who found in *Thraustochytrium aureum* ATCC 34304, DHA and DPA as major PUFAs.

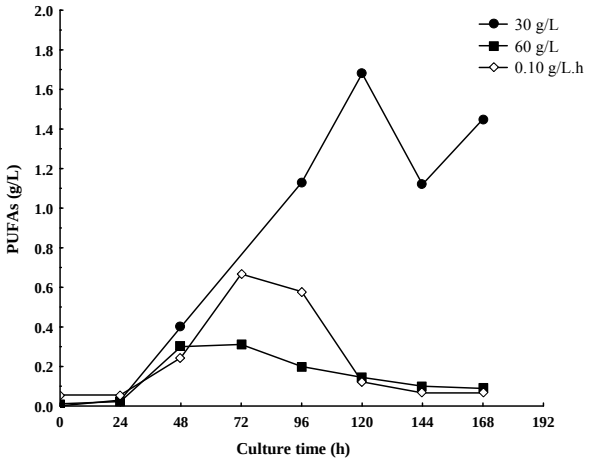


Fig. 4: PUFA content in biomass of *Thraustochytrium* sp., ATCC 26185, using glucose as carbon source

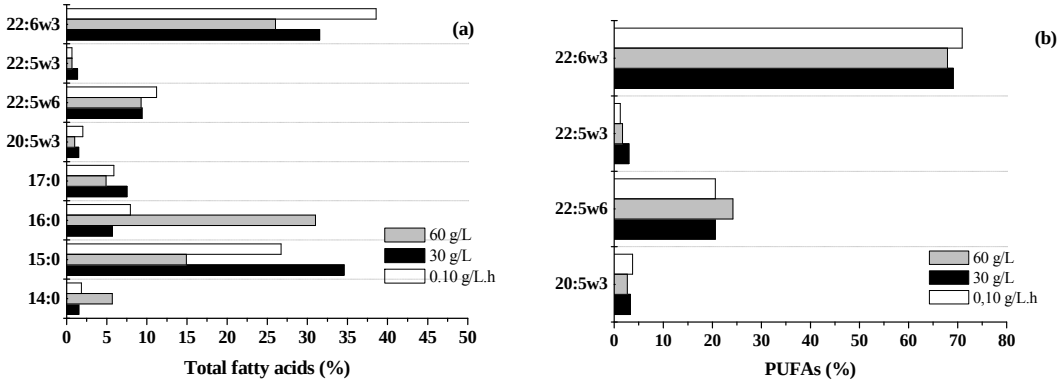


Fig. 5: Fatty acids composition of *Thraustochytrium* sp., ATCC 26185 cell biomass for 120 h (glucose 30g/L) and 72 h (glucose 60 g/L and 0.10 g/L.h)

In the experiment which used 30 g/L glucose (120 h of culture), 10.5% (w/w) of cell biomass is composed of PUFAs and 21% of these PUFAs are DPA ω-6 this is 2.2% of the total biomass (0.35 g/L). It can also be observed that 69% of PUFAs are DHA this is 7.3% of the total biomass (1.16 g/L). Jiang *et al*. (2004), observed a DHA content of 3.9% from *Thraustochytrium* sp. ATCC 26185, cultured for 120 h in darkness.

In the culture which employed 60 g/L glucose after 72 h, 6.9% (w/w) of cell biomass is composed of PUFAs and 24% these PUFAs are DPA ω-6, this is 1.7% of the total biomass (0.07 g/L). It can also be observed that 68% of PUFAs are DHA, this is 4.7% of the total biomass (0.19 g/L). In the culture that provided 0.10 g/L.h glucose (72 h point), 6.1% (w/w) of cell biomass is composed of PUFAs and 21% of these PUFAs are DPA ω-6 that is 1.2% of the total biomass of oleaginous microorganism (0.12 g/L). It can also be observed that 71% of these PUFAs are DHA that is 4.2% of the total biomass (0.42 g/L). Analyzing the majority fatty acids which constitute the PUFAs in the biomass, regardless of the type and culture time, the distribution of DPA ω-6 (21-24%) and DHA (68-71%) was approximated with little variation (Fig. 5b).

For as much as the experiment with 60 g/L glucose accumulated greater amount of PUFAs (6.9% w/w) in relation to the fed-batch (6.1% w/w), this had lower final yield of DHA (0.19 g/L), since besides the production of DHA being dependent on the accumulation of lipids and hence of PUFAs in the biomass, the yield is also related to the amount of biomass accumulated by this microorganism in a given time. Singh and Ward (1996b), achieved a maximum production of biomass and DHA of 17.1 and 2.0 g/L, respectively, supplementing with nutrient the *Thraustochytrium roseum* ATCC 28210 culture from the 4th day at intervals of 2 days. However these results, were obtained after 288 h of culture. On the other hand, the productivity (0.0069 g/L.h DHA)

obtained by those authors was lower than that achieved in this study (0.0097 g/L.h DHA) in culture with 30 g/L glucose.

In the study by Ka (2004), with the same strain, *Thraustochytrium* sp., ATCC 26185 and initial glucose concentration of 20.2 g/L, the greatest production of DHA was 0.23 g/L at 96 h. Furthermore, Taoka *et al*. (2011) reported that DHA content was 0.24 g/L from *Thraustochytrium aureum* ATCC 34304 cultured in a medium whit 30g/L of glucose and Twenn 80 for 96 h.

CONCLUSION

The higher biomass production (30.2 g/L) was recorded in the experiment which used 30 g/L glucose as carbon source. The polyunsaturated fatty acids majority in biomass were DPA ω-6 (21-24%) and DHA (68-71%), whose percentages did not show large variations with the growth conditions. The experiment that used 30 g/L of glucose during 120 h of culture showed the highest DHA yield, 1.16 g/L, it is 7.3% (w/w) of the cellular biomass of *Thraustochytrium* sp., ATCC 26185. This study demonstrated that the growth of *Thraustochytrium* sp., ATCC 26185 and the PUFAs production, particularly DHA, was dependent on the concentration of the carbon source available for its consumption as well as the culture period. Therefore, parameters such as medium composition and environmental factors should be considered to increase the production of PUFAs. However, studies will be necessary to investigate the concentration of nitrogen source available to that cell lipid producing microorganism.

ACKNOWLEDGMENT

This study was supported by Coordination for Improvement of Higher Education Personnel of Brazil (CAPES) and developed at the National Institute of Biological Resources (INRB/IPIMAR) in Lisbon, Portugal, through a scholarship granted to the first author by the Doctoral in the country with Internship Abroad Program-PDEE (Proc. Nº6906/10-9). The authors also thank Project ALGAENE and Depsiextracta Technologias Biologicas, Lda.

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