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DHA production by *Schizochytrium limacinum* SR-21 using crude glycerol as carbon source

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Abstract

This study investigates the potential of low-cost substrates (crude glycerol) usage as carbon source for obtaining docosahexaenoic acid (DHA), through fermentation of the *Schizochytrium limacinum* SR-21 microalga. The fermentations were conducted on flask and bioreactor scale. To quantify the amount of DHA obtained, the amount of biomass and lipid production were monitored and simultaneously running fermentative processes on two substrates were carried out. In both processes (flask and bioreactor) the highest amount of dry biomass (DB) was obtained by using glucose as carbon source (6.9 g L^{-1} in flask and 10.65 g L^{-1} in bioreactor). Although the amount of DB was higher on blank substrate, the level of DHA from total lipids content was higher (27.69 % in flask and 36.06% in bioreactor) in the biomass obtained on glycerol as primal carbon source. The highest quantity of DHA was obtained by using crude glycerol as carbon source for the microalgae when carrying out the process on bioreactor scale, which allowed us to control the pH on a set value of 7.5

Key words: crude glycerol, DHA, microalgae, substrate, *Schizochytrium limacinum.*

Polyunsaturated fatty acids (PUFAs) are a group of fatty acid containing two or more double bonds (Jasuja N.D. *et al*, 2010). Chemically, docosahexanoic acid (DHA 22:6) is an important PUFA, with a 22-carbon chain and six double bounds (chemical structure is presented in *Figure 1*), being considered a long chain polyunsaturated fatty acid (LC-PUFA) (Nagano N. *et al*, 2009; Jasuja N. D. *et al*, 2010; Boelen P. *et al*, 2013; Valenzuela A., and Valenzuela R., 2013).

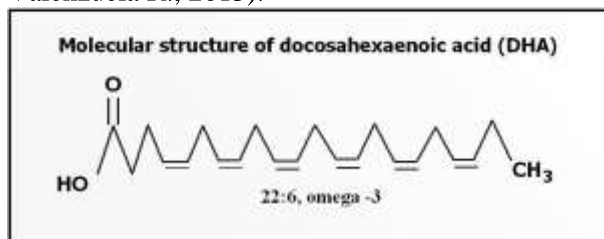

Figure 1 **Molecular structure of DHA 22:6**
(Valenzuela, A., Valenzuela, R., 2013)

In human body, DHA has a structural role, being an essential component of cell membrane in some tissues like brain and retina. (Zeng, Y. et al, 2011; Song, X. et al, 2015). Beside its structural role, DHA also confers some benefits to human health, lowering the incidence of coronary artery diseases, diabetes, inflammatory disorders, neuropsychiatric disorders (cognitive decline, Alzheimer, depression, schizophrenia) and other cardiovascular diseases (Yaguchi T. *et al*, 1997; Chin, H. *et al*, 2006; Chatdumrong, W. et al, 2007; Chi Z. *et al*, 2009). Unfortunately, the humans are not capable of synthesizing *de novo* the docosahexanoic acid, due to the lack of the enzymes responsible for *n*-3 PUFAs synthesis. Therefore, the only source of DHA is the dayly diet, which has an important role, especially for infants. (Zhu L. *et al*, 2008).

Nowadays, the largest source of commercial DHA is represented by the oil of few marine fish species: cod, salmon, mackerel, menhaden and tuna (Zhu L. *et al*, 2008; Ye C. *et al*, 2015). The fish oil is rich in DHA, but it exhibits two major disadvantages: the undesirable fish smell of the product and the risk of mercury (Hg) contamination because of the increasing pollution levels of oceans (Yaguchi T. *et al*, 1997). Due to the large market demand and limited fish resources, the scientists had been

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trying to find alternatives for replacing conventional sources of DHA (Huang T.Y. *et al*, 2012). For this purpose, the ability of a large number of microorganisms to metabolize cheap carbon sources has been tested. Thus, have been identified some microalgae species that are able to produce large amounts of DHA and other PUFAs if the growth process is conducted on specific medium (Yokochi T. *et al*, 1998; Chin, H. *et al*, 2006). In the case of microbial docosahexanoic acid few advantages were established. Firstly, the fatty acids obtained can be consumed by all categories of people, especially by vegans. Secondly, the obtaing process can be cheaper by replacing the conventional carbon (such as glucose) and nitrogen sources (different types of peptone) from the growth medium with wastes from different industries, reducing also the pollution level. Thirdly, by supplying the market with microbial DHA, the fish oil demand will decrease, protecting the oceans fauna.

Studies has showed that *Schizochytrium limacinum* SR-21 can produce large amounts of DHA, compared with in the biomass obtained for other microorganisms using glycerol as primal carbon source. (Yaguchi T. *et al*, 1997; Chatdumrong W. *et al*, 2007). *S. limacinum* is a marine microalgae, with unicellular structure and round shape cell, being part of thraustochytrid order (Jasuja N.D. *et al*, 2010). *Schizochytrium limacinum* was firstly isolated from the coral reef area of the Yap islands in the Federated States of Micronesia (Chin H. *et al*, 2006). This microalgae specie is capable to use glycerol as carbon source, making the DHA obtaining process economically efficient because the price of glycerol is under 0.1 $ kg^{-1} (Chi Z. *et al*, 2009; Abad S., Turon X., 2015; Lung Y.T. *et al*, 2016).

Glycerol is the main by-product of the biodiesel industry. For each 10 L of biodiesel, 1 L of crude glycerol is obtained (Abad S., Turon X., 2015; Lung Y.T. *et al*, 2016).

The purification costs of this by-product are very high, being economically inefficient process. The biodiesel comes like an alternative for substituting fossil fuels, because of their limited reserve. Since the industrial revolution began in eighteenth century, the fossil fuels demand and consumption increases annual. Burning of this fuels for energy caused one of the major global issues: global warming (Quispe C.A. *et al*, 2013; Lung Y.T. *et al*, 2016).

The present study has attempted to exploit the capacity of *Schizochytrium limacinum* SR-21 to use crude glycerol as carbon source for lipids production with a large range of DHA

MATERIAL AND METHOD

Schizochytrium limacinum SR21 (ATCC MYA-1381) was purchased from American Type Culture Collection and used in the present study. The cells were activate in 10 mL of artificial seawater (ASW) containing: 20 g L^{-1} glucose (VWR Chemicals, Belgium), 10 g L^{-1} yeast extract (Alfa Aesar, Karlsruhe, Germany) and 20 g L^{-1} sea salts (Sigma-Aldrich, USA), the composition of ASW being reported by Chin, H. et al in 2006. The medium pH was adjusted to 7.5 with NaOH solution (20%) before being autoclaved at 121 °C for 15 min. *S. limacinum* was incubated at 25 °C on an orbital shaker set to 150 rpm for 48 hours. After 48 hours 10 % (v/v) of the new culture was used to inoculate one 250 mL Erlenmeyer flask containing 50 mL of ASW. The flask was incubated in the same conditions as above, the new inoculum obtained being used for further fermentation processes.

For flask scale fermentation two different culture media were used. First medium (control medium) composition was: 20 g L^{-1} glucose, 10 g L^{-1} yeast extract and 20 g L^{-1} sea salts and the second medium contained: 90 g L^{-1} crude glycerol, 20 g L^{-1} sea salts and 5 g L^{-1} glucose. Flask scale fermentation was performed in 250 mL Erlenmeyer flasks containing 150 mL of each medium. The fresh media were inoculated with 10% (v/v) inoculum obtained previously and the fermentation process was carried out at 25 °C on an orbital shaker at 150 rpm for 120 h. Samples were collected in the beginning and the end of the fermentation for DHA and biomass monitoring.

On large scale, fermentations were carried out in a laboratory stirred tank bioreactor (Elecrolab, Fermac 300) equipped with a 2.9 L vessel volume and a digital control unit. Different fermentative processes were performed using both culture media described in the case of flask scale fermentations. The stirred tank was autoclaved at 121 °C for 15 minutes, cooled down at 25 °C and inoculated with 10% (v/v) inoculum. The temperature was set to 25 °C, the agitation speed at 150 rpm and the pH value was maintained at a specific set point of 7.5 using NaOH sterile solution (20%) as the correction agent. For biomass determining, aliquots of the fermentation liquid were taken once at 24 hours. In the final day, one sample was used for evaluation of total DHA content from biomass.

The samples collected from all of the fermentative processes (beginning and ending of flask scale fermentations and every 24 h aliquots from bioreactor fermentations) were centrifuged at 6500 rpm, 15 minutes and 4 °C. After centrifugation, the pellet was washed using 0.9 % NaCl solution and weighed. The dry biomass was determined by weighing the mass of the pellet after drying at 103 °C until constant weight.

For DHA analyses total lipids (TLs) were extracted from 5 g of wet biomass. The sample was homogenized in 20 mL of methanol for 1 minute with a high power homogenizer (MICCRA D-9, Germany) and 40 mL of chloroform were added, continuing the homogenization process for another 2 minutes. The mixture was filtered and the solid residue was reextracted two times with a mixture of chloroform/methanol (2:1 v/v). All the filtrates were mixed and washed with a 0.88% potassium chloride water solution and a methanol/water solution (1:1 v/v). The purified lipid layer was dryed over anhydrous sodium sulfate and the solvent was removed in a rotary evaporator, obtaining the TLs fraction was obtained. The extraction method was reported by Dulf F.V. *et al*, (2013).

The TLs were methylated using acid-catalysis transesterification procedure and the fatty acid methyl esters (FAMEs) were obtained. The FAMEs were analyzed by gas chromatography-mass spectrometry (GC-MS) using a PerkinElmer Clarus 600 T GC-MS (PerkinElmer, Inc., Shelton, CT, USA) equipment. The entire process (transesterification and GC-MS analysis) was carried out following the procedure described by Dulf F. V. *et al* (2013).

RESULTS AND DISCUSSIONS

The present study represents an investigation of the influence of carbon source on DHA production by fermentation processes. The carbon sources used were glucose and crude glycerol. Through the fermentative processes, the DHA can be produced by microalgae *S.limacinum* SR-21 with it's ability to metabolize glycerol, at a laboratory scale process performed in optimal conditions. In order to improve the DHA production we tried to find the optimum culture conditions of *S. limacinum*. It has been proven that the amount of glycerol has an important role in the production of DHA at the laboratory scale, since a large amount in medium inhibits the growth of the microalgae. In order to determine the optimum amount of crude glycerol needed for *S. limacinum* SR-21 to produce DHA several experimental trials were performed.

Following the experimental trials was concluded that the optimum value of glycerol concentration is 90 g L^{-1}, as confirmed by similar results reported by Chi Z. *et al*, (2009). Because the inoculum was obtained in a medium containing glucose and the enzymatic system of the microalgae was set to metabolize this carbon source, besides of 90 g L^{-1} of crude glycerol, 5 g L^{-1} of glucose were needed to facilitate *S.limacinum* SR-21 growth.

First fermentation processes were carried out in flasks, where the pH wasn't monitored during the 120 hours. *Figure 2* presents the quantity of dry biomass (DB) at the beginning and at the end of the processes, expressed in g L^{-1}. For the fermentation carried out on glucose as carbon source, the DB quantity was higher (6.9 g L^{-1}) than the quantity obtained in the case of crude glycerol (6.6 g L^{-1}).

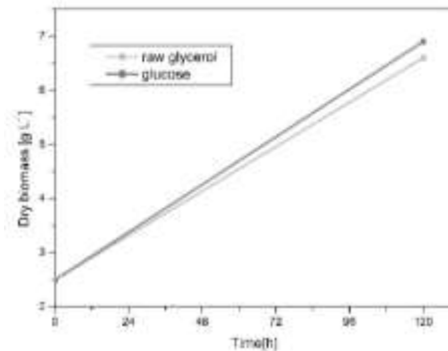

Figure 2 **Dry biomass (DB) evolution in flask scale fermentations**

After obtaining the results on flask scale, the fermentations were carried out on bioreactors, in which case the pH was maintained on a specific set point of 7.5. Consequently, the biomass quantity was higher than in the case of flask fermentations where the pH wasn't monitored. *Figure 3* shows the evolution curve of the dry biomass level during the fermentative processes in the bioreactor. At time 0 the DB quantity was 2.5 g L^{-1} for both carbon sources, highlighting the fact that in the first 24 hours of the fermentative process the quantity increased with 1.6 g L^{-1} for the medium containing crude glycerol and 1.3 g L^{-1} for the medium with glucose. One explanation can be that the medium with glycerol was enriched with a small amount of glucose, containing two carbon sources in the same time. The highest increase was recorded between the 48th and 96th hours of the fermentation, the concentration of microalgal cell being almost constant on both media after that moment. As shown *Figure 3* the quantity of dry biomass in last day of fermentation was 10.65 g L^{-1} on glucose substrate and 9.12 g L^{-1} on crude glycerol. For both processes (flask and bioreactor) the amount of dry biomass was higher using glucose as carbon source.

Comparing our quantity with the dry biomass amount which Chin H. *et al*, (2006) reported in their study, can be noticed that is a small difference of biomass level using same concentration of sea salts, same temperature and same time of fermentation that we used. One reason for this small difference can be the concentration of glucose which was used. Chin H. *et al*, (2006) used a double concentration of glucose as carbon source (4%). The biomass

quantity in their study was 7.27 g L^{-1} while we obtained in our study 6.9 g L^{-1} after 5 days.

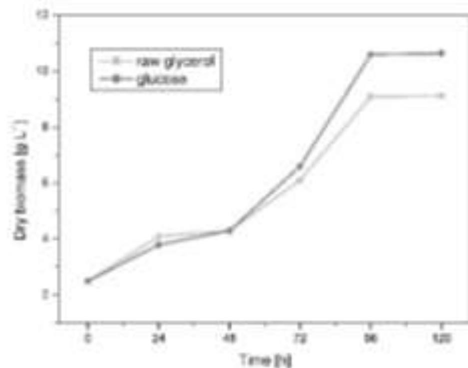

Figure 3 **Dry biomass (DB) evolution in bioreactor scale fermentations**

This results were obtained by carrying out the processes on flask scale. Comparing with our results it can be concluded that with a half concentration of glucose but same parameters the difference wasn't substantial. By performing the fermentations in bioreactor and maintaining the pH at a set point of 7.5 during the entire process the biomass level was increased to 10.65 g L^{-1}. Also, by replacing 75% of glucose quantity with 90 g L^{-1} crude glycerol (which is a cheaper carbon source) and performing the process on bioreactor scale, the biomass amount was higher (9.12 g L^{-1}) than the one Chin H. *et al*, (2006) obtained in their study.

In the end of both processes (flask and bioreactor fermentations) the medium was centrifuged and the wet biomass (WB) was separated. For DHA analyze from WB, total lipids (TLs) were extracted and transesterified into fatty acids methyl esters (FAMEs). FAMEs were analyzed with a gas chromatograph and the fatty acids profile was obtained.

The percentage of DHA from total fatty acids (TFA) obtained by GC-MS analyses of FAMEs is presented comparatively for both processes in *Table 1*.

As shown in *Table 1* there are sizeable differences between flask scale fermentations and bioreactor trials regarding the DHA content from TFA. By controlling the pH value and maintaining at a set point of 7.5, the DHA productivity has been increased. Although the amount of biomass was higher by using glucose as carbon source, the DHA level was bigger in the biomass obtained by using crude glycerol. The highest percentage of DHA from TFA was obtained by using crude glycerol and caring the fermentation in bioreactor (36.06%) and the lowest level of DHA was in the case of flask fermentation using glucose (25.34%).

Table 1

Fatty acids content of TLs extracted from microalgae *S. limacinum* SR-21

Type of fermentation	Carbon source	Fatty acid content (%)																
		12:0	13:0	14:0	14:1 n-3	15:0	16:0	16:1 n-9	16:1 n-7	17:0	18:0	18:1 n-9	18:2 n-6	18:3 n-6	18:3 n-3	20:5 n-3	22:4 n-6	22:6 n-3
Flask	Glucose	0.38	0.06	2.54	0.09	11.49	43.49	0.14	0.17	1.50	1.60	0.81	0.66	0.17	0.42	0.82	10.32	25.34
	Glycerol	0.28	0.19	6.85	0.42	8.83	41.54	0.03	0.24	1.88	1.34	0.20	0.09	0.19	0.17	0.47	9.59	27.69
Bioreactor	Glucose	0.33	0.18	2.34	0.13	13.30	31.45	0.24	0.16	5.92	2.08	1.41	1.01	0.41	0.32	0.65	8.81	31.25
	Glycerol	0.32	0.12	7.51	0.19	5.11	40.75	0.02	0.08	0.91	1.22	0.03	0.02	0.08	0.07	0.32	7.20	36.06

Making a comparison between the results obtained by Chin H. *et al*, (2006) and our results

for flask scale fermentations, it can be noticed that there aren't any substantial differences.

By carrying out the processes in bioreactor and using crude glycerol as major carbon source, the DHA concentration from TFA was higher (36.06%) than the one Chin H. *et al*, (2006) obtained using same microalgae strain.

CONCLUSIONS

In conclusion, the present work demonstrates that *S. limacinum* SR-21 is capable of crude glycerol metabolizing. Using a low cost medium (containing crude glycerol, sea salts and water) and controlling the pH value in a bioreactor, the level of DHA from TFA was increased. Crude glycerol is a cheap carbon source which can serve as a substrate for industrial DHA producing. By implementing the fermentative process on large scale and, can be solved one of the biggest problem of biodiesel industry: huge cost of purification process of crude glycerol (the main by-product).

ACKNOWLEGMENTS

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