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Improvement of Biomass and Dha Production on a Semi-Continuous Culture of *Aurantiochytrium* sp NYH-2

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Aurantiochytrium sp has been recognized as one of the most suitable sources of high valuable fatty acids (FA) including docosahexaenoic acid (DHA), docosapentaenoic acid, arachidonic acid, eicosapentaenoic acid (EPA), and saturated FA; however it's necessary to improve total biomass production and high value FA specially DHA.
The aim of this research is to improve the production of biomass and DHA on the novel *Aurantiochytrium* sp NYH-2 strain via an optimization of culture time (2, 3 and 5 days) and removal of culture (20, 50 and 80% v/v) using a 2^3 experimental design.
It was found that in order to improve biomass production a large amount of culture media must be replaced (>90%), however after the third cycle biomass production was reduced from 10 g/L to 9 g/L; similarly under the same conditions lipids and DHA production increased from 15% to 20% (w/w) and from 3 to 5% (w/w) respectively in the first 3 cycles and then reduced in cycle 5. Finally results suggest that in a semi-continuous process the algae is only functional 3 cycles (9 to 12 days) and in order to improve total biomass lipids and DHA production, a volume of culture media between 80-92% must be removed every 3-4 days.

1. Introduction

Microalgae have received much attention over the last 20 years, not only because of its biotechnological potential but also for its environmental and ecological relevance (Lee Chang et al. 2013); over the years research has focused heavily over some genera and species which have shown great industrial and scientific interest, however there is great diversity still undetermined, an example of the above are the Thraustochytrids (Labyrinthulomycetes), these microorganism are obligate heterotrophic fungus-like protists that can be found ubiquitously in the water column and sediments on the marine environment (Barclay et al. 1994), and even thought being non-photosynthetic (Armenta and Valentine 2013) in fact are considered "algae" based on the molecular evidence suggesting that branch from the primary position of Heterokontophyta, which include brown algae and diatoms. Thraustochytrids play an important role in the marine ecosystem due to its ability to act as bacterivores, detritivores, and parasites (Maas et al. 1999; Mo et al. 2002; Raghukumar 2002). Thraustochytrids settle on fallen leaves, and fecal pellets of zooplankton and carry out extracellular enzymatic decomposition of complex organic substrates for the absorption of nutrients (Findlay et al. 1986; Bremer 1995), in some places (specially coastal waters and sediments) has been found high densities, this findings suggest that these microorganisms play a big role on the carbon turnover (Santangelo et al. 2000; Bongiorini et al. 2004); also Thraustochytrids are important component of the detrital food web, due to its natural high content of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and Eicosapentaenoic acid (EPA); due to this special characteristics Thraustochytrids are recognized as a novel biotechnological source for the production of high value products (Kobayashi et al., 2011). DHA has received much attention due to their beneficial effects on neural development of infants, reduction of cardiovascular events, hypertension, arthritis, sclerosis and thrombosis (Ren et al, 2014; Sun et al., 2014), thanks to this global market has expanded significantly and it is expected that by 2016 the sale of DHA reach a value of $ 34.7 billion. For the production of DHA the preferred carbon source is glucose, but glucose for oil

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is very expensive, due to two factors: high cost of this carbon source and low conversion into DHA (10-15%), because of this in recent years research has focused on the implementation of low-cost carbon sources and the optimization of variables such as culture time and nutrient concentration. The aim of this research is to improve for the first time the production of biomass and DHA on the novel Aurantiochytrium sp NYH-2 strain via an optimization of culture time and removal of culture.

2. Materials and methods

2.1 Microalgae culturing

Aurantiochytrium NYH-2 was isolated from a mangrove coastal area in Okinawa Prefecture, Japan. The organisms were maintained on agar plates containing modified GTY agar plates containing 20 g glucose, 10 g tryptone, 5 g yeast extract, 18 g of natural sea salts.

2.2 Design of Experiments

In order to test the effect of culture time and culture removal a 2^3 Central composite Design (Table 1) was applied using STATISTICA® 7.0. For each test the alga was precultured in 500 mL conical flasks containing 200 mL of GTY liquid medium with rotational shaking (100 rpm) at 25°C for three days. After 3 days 1 mL was inoculated on 200 mL of fresh GTY medium and cultured according with the design of experiments, after the specified time for each experiment is complete, an specific amount of medium for each experiment was removed and replaced with fresh medium, this process was repeated until completing 4 cycles.

Table 1: Variables obtained for the Design of Experiments 2^3

Exp	1	2	3	4	5(C)	6	7	8	9	10(C)
Culture removal (% v/v)	20	20	80	80	50	7.57	92.4	50	50	50
Culture time (days)	3	5	3	5	4	4	4	2.6	5.4	4

2.3 Lipids analysis

After each cycle, an specific amount of media was removed and biomass was harvested by centrifugation (3400 rpm, 20 min, 20ºC) rinsed twice with distilled water and then lyophilized and weighed. The total amount of lipids and fatty acids were measured according to Nakazawa et al (2012). Briefly, 50 mg of dried alga was inmersed in chloroform:methanol (2:1 v/v). Fatty acids were transmethylated from the extract using 14% BF_3 and the esterified fatty acids were extracted using n-hexane. The final extracts were applied to a GC-FID (Shimadzu GC-2010) equipped with a DB-5MS capillary column (30 m 0.25 mm inner diameter; 0.25 mm film thickness; J & W Scientific, Agilent Technologies Japan, LtD). The temperature program rose from 130°C to 270°C with increments of 20°C/min. Peaks were identified using authentic standards of fatty acid methyl esters.

3. Results and discussion

Results for biomass production (figure 1) shown that culture times no larger than 4 days coupled with larger exchange of culture media will improve biomass concentration up to 10 g/L (productivity of 2 $g/L*d^{-1}$), these results agree with those reported by Hong *et al* (2011) and Lee Chang *et al* (2013) where high initial concentrations of glucose boost biomass and lipid production durign the first 49 hr; however, substrate glucose levels as high as 100 g/L resulted in the retardation of biomass production in *Aurantiochytrium* strains (Yaguchi et al. 1997; Wong et al. 2008).

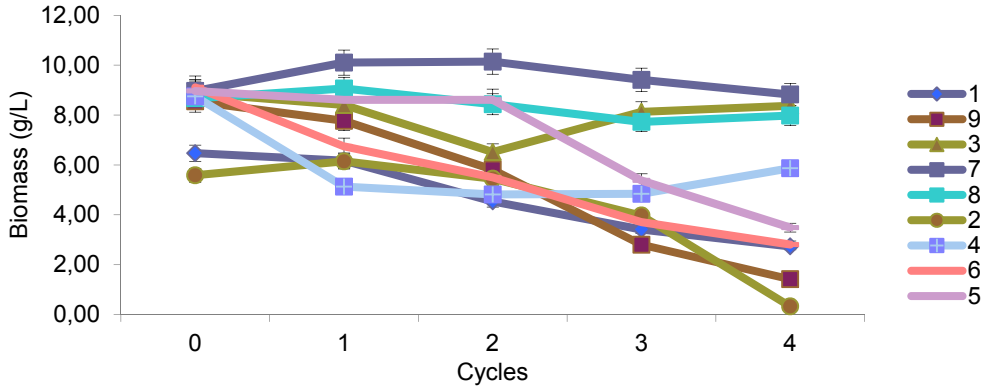

Figure 1: Biomass production over 4 cycles of culture exchange

According to the Pareto chart (Figure 2, right), the most significant variable is the culture removal, moreover the response surface (Figure 2, left) shows that larger biomass concentration values requires larger culture media exchange (up to 90%) and production times between 2-3 days.

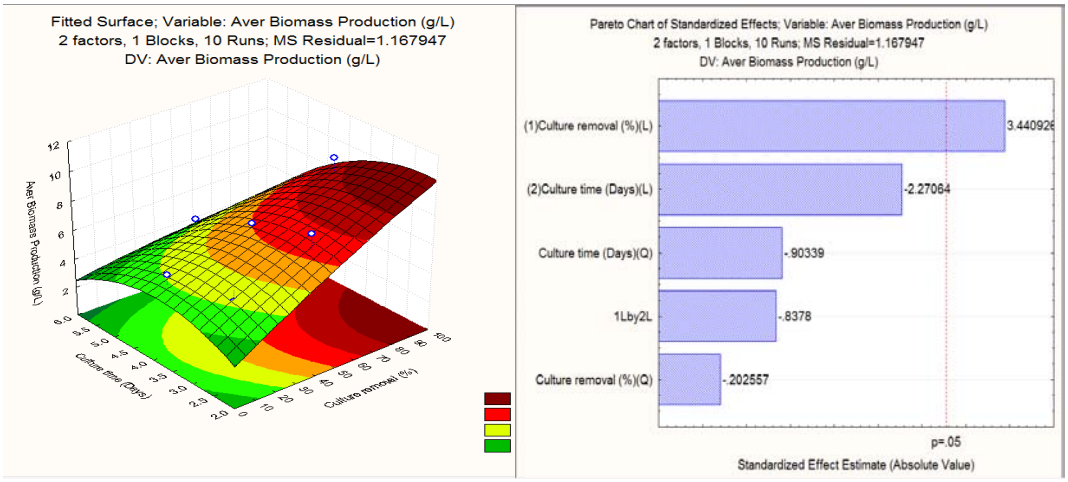

Figure 2. Surface response (left) and Pareto Chart for biomass production

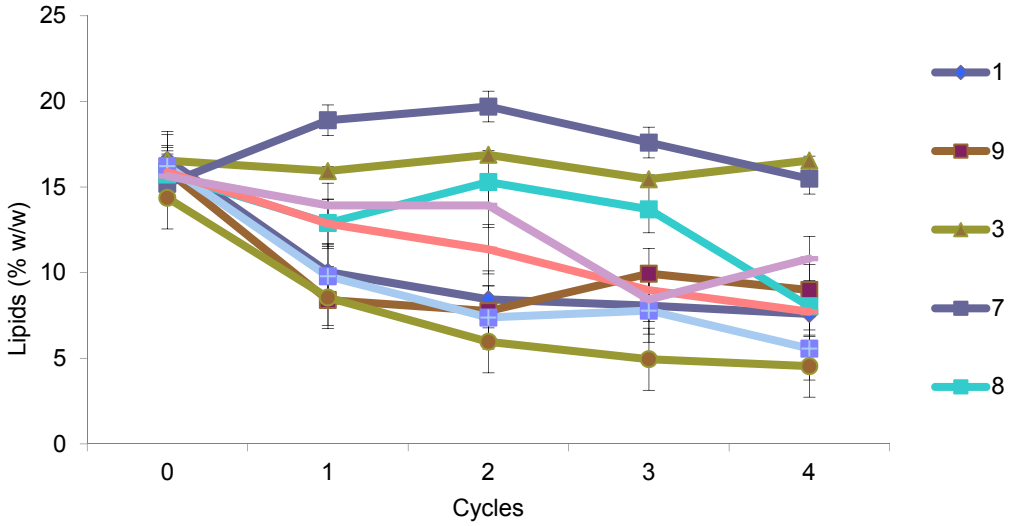

Figure 3: lipids production over 4 cycles of culture exchange

Results for Lipids concentration (figure 3) shown that culture times no larger than 4 days coupled with larger exchange of culture media will improve lipid concentration up to 20% w/w; however, after cycle 2 the lipid concentration decreased considerably up to 17% on cycle 3 and finally 15% (w/w) on cycle 4. According to Pareto chart (Figure 4, right) the most significant variable is the culture removal, moreover the response surface (Figure 4, left) shows that larger biomass production values requires larger culture media exchange (up to 90%) and production times between 2-3 days.

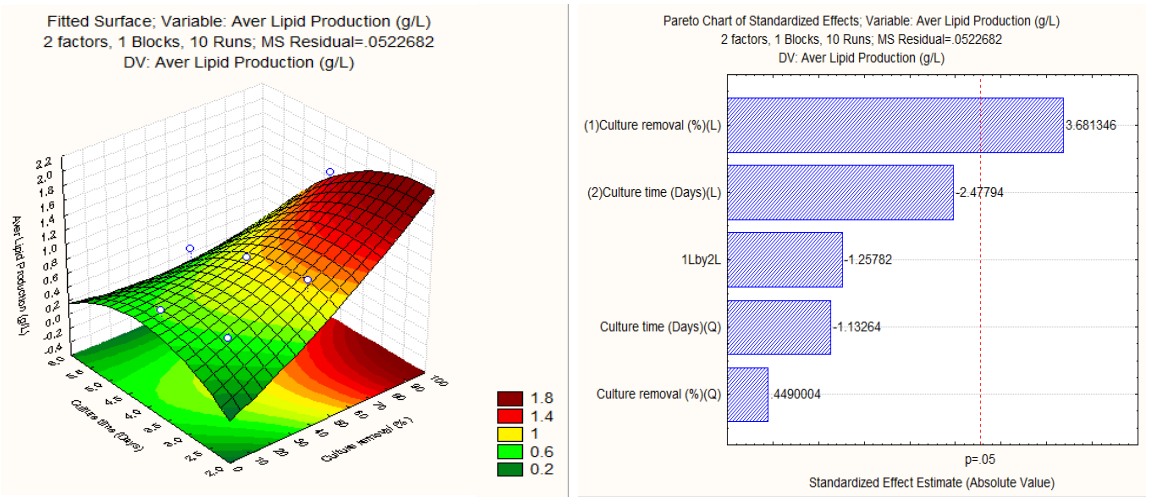

Figure 4. Surface response (left) and Pareto Chart for lipids production

The literature for different *Aurantiochytrium* strains focus on optimizing the potential sources of carbon, nitrogen, and trace element concentrations and other variables such as temperature, pH and agitation; however few studies focused on determining the possible maximum culture time for this alga. Studies such as those developed by Lee Chang (2013) have shown that in fed-batch systems *Aurantiochytrium* can grow for 3 days, others as Gao *et al* (2013) have produced up to 6 days; however, Huang *et al* (2012) found that after 5 days the production of biomass and DHA decreased instantly.

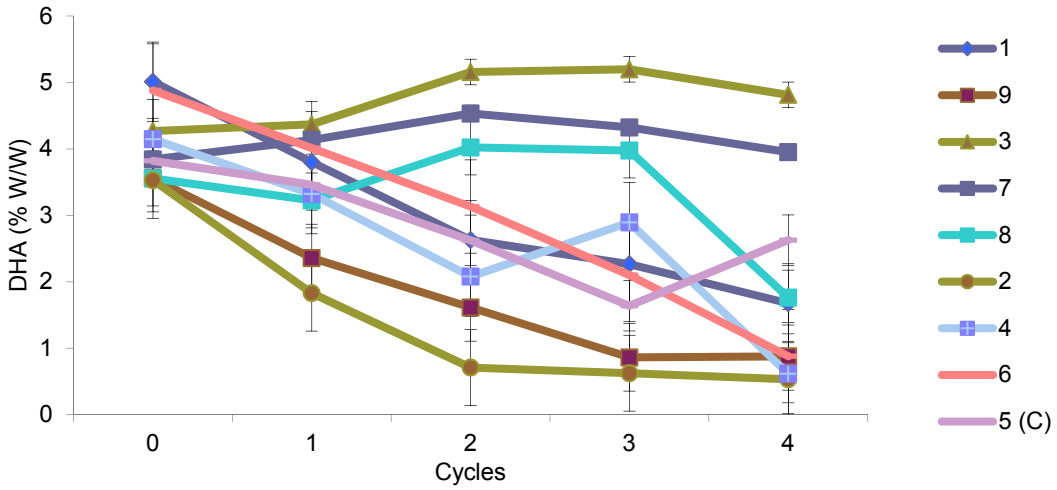

Figure 5: DHA production over 4 cycles of culture exchange

Unlike the variables for the increase of biomass and lipids concentration, DHA synthesis (figure 5) requires between 3 to 4 days of culture and a exchange of media up to 90%; furthermore another difference between DHA and total lipids lays on the Pareto chart (figure 6, right) which shows that the culture time is a significant variable

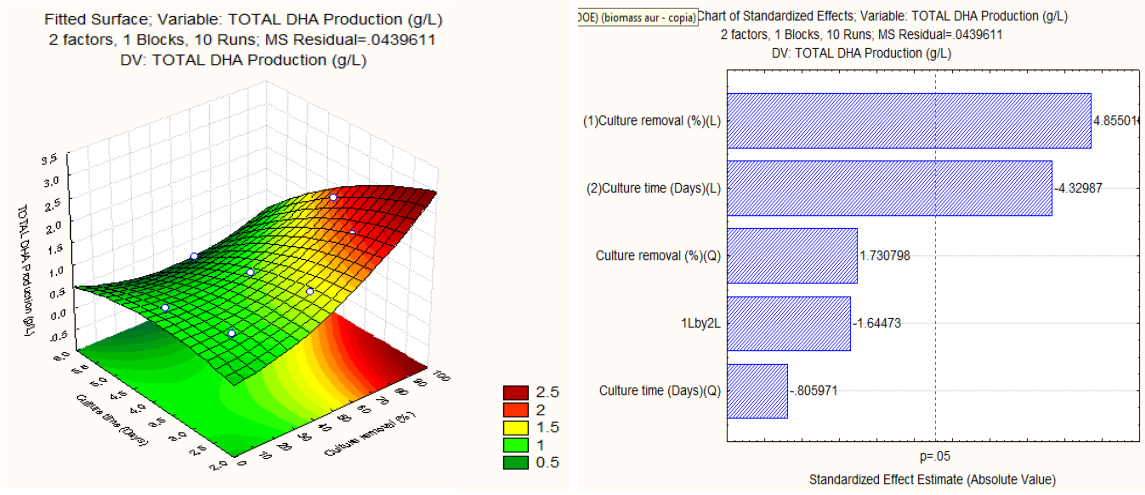

Figure 6. Surface response (left) and Pareto Chart for biomass production

This is the first experiment were exhausted media of *aurantiochytrium* sp is removed and changed by fresh media culture, normally this algae has been tested on batch or fed-batch cultures; howev media is removed fromIn a semi-continuous process, the algae is only functional 3 cycles (including cycle 0), also this work presents for the first time a culture process that exceeds 6 days of cultivation. Wu & Lin (2003) found that using 27,98 g/L glucose 4,52 g/L yeast extract and 4 days of culture the DHA concentration can be 0,516 g/L (Eficiency of 3,68%) In contrast, this study found a DHA concentration of 0,46 g/L using 18 g/L of glucose, 5 g/L yeast extract and 4 days (Eficiency 4,31%), this is a significant reduction in the concentration of glucose needed without affecting production time (4 days).

4. Conclusions

Results allow to conclude that in order to maintain a stable culture production of *Aurantiochytrium* sp with a biomass concentration of 8-10 g/L, 15-20% w/w of lipids and up to 5% w/w of DHA, a culture volumen between 75-90% (v/v) must be removed every 2-3 days over a total time of 12 days, because after cycle 4 even when almost 90% of fresh media is added the alge will not grow, this still remains as an important barriers which should be studied more carefully in order to understand the biochemical processes that limit this alga.

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