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Improvement of medium composition and cultivation conditions for growth and lipid production by *Crypthecodinium cohnii*

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Abstract

In this study, glycerol was used as an alternative carbon supply in the growth medium of Crypthecodinium cohnii. Concentration of other nutrients was optimized by using response surface methodology (RSM) for the growth and lipid production by microalgae. As a result, when using yeast extract, there was no need using glutamic acid and sodium β-glycerophosphate in the growth medium. Additionally, it was revealed that there was a reduced growth by C. cohnii with a certain yeast extract concentration. The optimum sea salt concentration in the medium was determined as 16 g L^{-1}. In the optimized medium, the highest biomass productivity and total lipid content achieved were 1.0 g $L^{-1}d^{-1}$ and 30 % of dry weight basis, respectively. The yield coefficient of $Y_{X/S}$ on glycerol obtained in this study was between 0.30-0.48 (w w^{-1}). The effect of mechanical agitation on the growth and lipid production by C. cohnii was investigated in a 2 L stirred tank bioreactor. Nile red based flourescence intensity measurements during bioreactor productions revealed that lipid production by C. cohnii continued even after cells entered the stationary phase. The docosahexaenoic acid proportion increased until the beginning of stationary phase (5^{rd} day) at 150 rpm agitation rate in bioreactor.

Keywords: microalgae, optimization, heterotrophic culture, batch processing, lipid fluorescence intensity

1. Introduction

Medium composition, especially carbon, nitrogen and phosphorous sources, is one of the critical factors for influencing the microalgae growth, which can grow autotrophically or heterotrophically (SHEN & al. [1]; PEREZ-GARCIA % al. [2]; ISLETEN-HOSOGLU & al. [3]). Therefore, to obtain high biomass and lipid productivities, it is important to find appropriate types of nutrient sources and their optimum concentrations in growth medium of microalgae. *C. cohnii* is a promising microalga to be used as a primary source for docosahexaenoic acid (DHA). In the current process of *C. cohnii* cultivation, glucose is known as the principal carbon source (DE SWAAF & al. [4; 5]; MENDES & al. [6]). In productions performed by using different carbon sources (glucose, ethanol, acetic acid) in *C. cohnii* cultivation indicate that the cell growth and lipid accumulation were affected by different carbon sources

(DE SWAAF & al [4;5;8]; RATLEDGE & al. [7]). Glycerol can also be considered as a potential carbon source for the cultivation of *C. cohnii*. During the biodiesel production process, glycerol is produced as a by-product. Thus, researchers have been looking for ways converting glycerol into value added products (CHI & al. [9]; O'GRADY and MORGAN [10]). According to some researchers, *C. cohnii* could use glycerol (MENDES & al. [11]. However, others reported that no marginal growth was observed when glycerol was offered as a carbon source (DE SWAAF & al. [4]). Studies have not been performed to examine the effects of glycerol on cell growth, lipid production and overall lipid profile by *C. cohnii* in detail. Yeast extract was generally used nitrogen source with a wide range of concentration (1–11.5 g L^{-1}) (DE SWAAF & al [5]; MENDES & al. [6]; DA SILVA & al. [12]). Pleissner and Eriksen [13] used the glutamic acid as the main nitrogen source. Microalgae also modify their biochemical composition in response to salinity (JIANG and CHEN [14] and environmental changes, such as stirring rate in a bioreactor, temperature, pH (ISLETEN-HOSOGLU & al. [3]; JIANG and CHEN [14;15]).

Microbial bioprocesses are very complex and influenced by many factors and also their interactive effects. Thus, an appropriate experimental designs can be used to study the effects of various factors on the bioprocess to make it better understood and improve its performance (MANDENIUS and BRUNDIN [16]; WANG and WAN [17]). The traditional or statistical experimental designs can be used to make a choice between those different nutritional sources. Most of these reports present the effects of a single variable on the biomass productivity and the lipid pool of the microalgae where interactive effects of more than one variable might be important (DE SWAAF & al. [4]; JIANG and CHEN [15]; JIANG and CHEN [18]). Statistical experimental designs help understanding the interactions among ingredients, reduce the number of experiments resulting in saving money and time (MANDENIUS and BRUNDIN [16]).

Medium optimization carried out in this study was aimed to gain better understanding on the nutrient requirements for optimal growth and lipid production by *C. cohnii*. Also, batch cultures were carried out in a stirred tank bioreactor for investigating effects of some process factors on growth and lipid production by *C. cohnii*. Additionally, the measurement of lipid content of cells during the cultivation process in response to cultivation conditions might enable better understanding of the relationship between cell growth and lipid accumulation. In this study, the right time of cell harvest was monitored with the nile red based flourescence measurement.

2. Materials and Methods

C. cohnii CCMP 316 was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA. Cultures were maintained axenically in liquid ATCC medium 460 A2E6 medium with 3 g L^{-1} glycerol. Vitamins and trace metals were supplied as reported in the ATCC medium 460 A2E6. The medium, except carbon source, was sterilized in an autoclave at 121 ºC for 20 min. Stock cultures were inoculated into 100 mL medium in 250 mL Erlenmeyer flasks with glycerol as a carbon source and initial pH 6.5. The flasks were incubated at 24 ºC in an orbital shaker and agitated at 130 rpm in the dark.

Batch cultivations were also performed in a 2 L stirred tank bioreactor (STR) (Biostat B-plus, Sartorius, Melsungen, Germany) with 1.5 L working volume. The STR was equipped with two six-blade disk impellers (diameter 53 mm). A dissolved oxygen probe (Hamilton Oxyferm FDA 225, Bonaduz, Switzerland) and a pH sensor (Hamilton Easyferm K8 200, Bonaduz, Switzerland) were installed on the top plate of the

bioreactor. The culture of *C. cohnii* in the exponential growth phase was inoculated into the bioreactor at 10 % (v v^{-1}) level. The initial pH was set to 7.0 and it was controlled throughout the experiments. Aeration rate and temperature were set as 1 vvm, 24 ± 1 °C, respectively. There was no intervention on process parameters during the production.

Cell concentration (optical density) was estimated by absorbance of the suspension at 700 nm with a UV–Vis spectrophotometer (Ultrospec 1100 pro, Amersham Bioscience). A calibration curve between cell number and cell dry weight (g L^{-1}) was developed by filtering the aliquots on pre-weighed GF/C filter paper (Whatman, UK). The filtered cells were dried at 105 ºC until constant weight was obtained and cooled to room temperature in a desiccator before weighing

Samples for lipid analysis from shaken flasks were harvested by centrifugation at 1500 g for 5 min and washed at least twice with demineralised water. Prior to and after freeze drying, the samples were stored at –20 °C.

Oil was extracted from lyophilized algal biomass by a modified method of Bligh and Dyer [19]. Freeze-dried cells (100 mg or more) were weighed accurately into a 15 mL centrifuge tube. For extraction, 3 mL chloroform: methanol (2:1) containing 1.0 mg mL^{-1} nonadecanoic acid (19:0) and 0.5 mg mL^{-1} BHT were used and the tube was shaken gently overnight at room temperature. After centrifugation at 1500 g for 5 min, the supernatant containing the extracted oil was stored at 4 °C until analysis DE SWAAF & al. [5]. The extract was evaporated in a water bath (30 °C) using a rotary evaporator (Stuart, RE300,UK) to remove solvents.

Fatty acids were analyzed by gas chromatography (GC) after direct transmethylation with hydrochloric acid in methanol with small modifications by Christie [20]. The fatty acid methyl esters (FAMEs) were extracted with hexane and analyzed by Agilent 7890 gas chromatography equipped with a flame-ionization detector and a Supelco sp-2380 A capillary column (60m x 250µm x 0,2 µm) with helium as a carrier gas (ISLETEN-HOSOGLU & al. [21]).

The stock solution of Nile red (NR) (Sigma, N3013) was prepared in isopropanol. The same concentrations of NR and spectrofluorometer conditions were used for *C. cohnii* which was used before for the green algae *C. saccharophila* (ISLETEN-HOSOGLU & al. [21]). The lyophilized *C. cohnii* powders were diluted to appropriate concentration with phosphate buffered saline (PBS) just prior to flourescence measurement. Cellular concentration was determined by optical density. Effect of cell concentration on lipid quantification was investigated by mixing alga cell of different OD values 0.030-0.400 with the optimized NR concentration. Having stained algal cell suspensions, fluorescence was measured on a Varian 96-well plate spectrofluorometer (Varian, Cary Eclipse).

Lipid content versus fluorescence intensity was determined as a correlation between the NR fluorescence intensity and lipid content assayed by the Bligh and Dyer [19] extraction method, by analyzing at least six samples with different lipid contents.

The quantification of glycerol was performed by using a Thermo (Thermo Scientific Finnigan Surveyor RI plus detector) high performance liquid chromatography (HPLC) system. Samples were separated on a Phenomenex Rezex RHM Monosaccaride (H+) column (300 x 7.8 mm) maintained at 65 ºC. The mobile phase was 5 mM sulfuric acid with a flow rate of 0.8 ml min^{-1}. The injection volume was 15 µL (ISLETEN-HOSOGLU & al. [21]).

Response surface methodology (RSM) designs such as Box–Behnken and Central Composite Design (CCD) model probable curvature of the response function MANDENIUS and BRUNDIN [16]. Therefore, these methods of response surface design in Design Expert software (version 7.0.0, Stat-Ease Inc., Minneapolis, MN), were used to perform the experimental design for the optimization of carbon, nitrogen, phosphorus and sea salt concentrations in the growth medium of *C. cohnii*. First of all, a total of 16 runs Box–Behnken design were used to optimize the range and levels of the glutamic acid, sodium β-glycerophosphate and yeast extract in the growth medium (n=4). After completing the first design, a total of 20 runs CCD design were used to optimize the range and levels of the sea salt, glycerol and yeast extract in the growth media for microalgal growth and lipid production (n=6). For predicting the optimum points, second order polynomial functions were fitted to correlate relationship between independent variables and responses.

3. Results and Conclusions

There was a correlation between the cell number and the optical density at 700 nm, OD_{700} for *C. cohnii*. Additionally, a correlation was found between the cell number and the cell dry weight (g L^{-1}). The following regression equations, (Eq. (1), Eq. (2)) were obtained from measurements:

Cell number (*10^4 mL^{-1}) = 128,33OD_{700} $R^2 = 0.99$ (1)

Cell number (*10^4 mL^{-1}) = 89,023*cell dry weight (g L^{-1}); $R^2 = 0.99$ (2)

Our preliminary screening studies indicated that *C. cohnii* showed both better growth and lipid production on glycerol (data not shown) rather than glucose which was very commonly used carbon source (DE SWAAF & al. [4]; JIANG and CHEN [14]; JIANG and CHEN [18]. Therefore, glycerol was used as a carbon source throughout the optimization experiments. ATCC 460 A2E6 medium used as the basic medium for maintaining *C. cohnii* cultures contained glutamic acid as a nitrogen source and sodium β-glycerophosphate as a phosphate source. These nutrients are not commonly preferred for large scale microalgal productions because of the production cost. Generally used complex nitrogen source was yeast extract for heterotrophic microalgal cultivations (DE SWAAF & al. [5; 8]; O'GRADY and MORGAN [10]). The comparison of effect of three nutrition sources and optimization of their concentrations in culture medium of *C. cohnii* were accomplished by Box–Behnken model. Three variables (glutamic acid, sodium β-glycerophosphate and yeast extract), along with their low, medium and high levels, were used as the experimental design model for optimization of growth of *C. cohnii*. According to the implemented design, sixteen combinations were performed. In the experimental design model, glutamic acid (0.1–2.0 g L^{-1}), sodium β-glycerophosphate (0.15–0.90 g L^{-1}) and yeast extract (0.1–2 g L^{-1}) were taken as input variables. Cell dry weight (g L^{-1}) was taken as a response of the system. Total cell dry weight in different experimental conditions based on the experimental design matrix was estimated. A second order polynomial model obtained from the Box–Behnken model experiment can be stated in the form of the following equation:

$$Y = 3.15 + 0.011X_1 – 0.047\ X_2 + 1.43\ X_3 – 0.45\ X_3^2 \quad (3)$$

where Y is the predicted response, i.e. the cell dry weight (g L^{-1}), and X_1, X_2, and X_3 are the coded values of the test variables, glutamic acid, sodium β-glycerophosphate and yeast extract concentrations (g L^{-1}), respectively.

The statistical analysis of the model was accomplished in the form of analysis of variance (ANOVA). The model F-value of 170.18 indicated that the model was significant ($p < 0.01$). According to the ANOVA test, only the yeast extract had a significant effect on cell dry weight of *C. cohnii*. Therefore, glutamic acid and sodium β-glycerophosphate should be at their minumum level in the growth medium. In previous researches, the yeast extract concentration was varied in a wide range (1-11.5 g L^{-1}) (DE SWAAF & al. [4; 5; 8]; MENDES & al. [11]). In this study, it seemed that there was a reduced growth by *C. cohnii* with yeast extract concentration exceeding 2 g L^{-1}.

In a previous experimental design, it was revealed that glutamic acid and sodyum β- glycerophosphate should be at their minumum level in the growth medium of *C. cohnii*. Then, the concentrations of glutamic acid and sodyum β- glycerophosphate were kept at 0.1 g L^{-1} and 0.15 g L^{-1}, respectively. The most important nutrients affecting the growth and the lipid production in heterotrophic microalgal cultures are nitrogen and carbon sources (PEREZ-GARCIA & al. [2]; ISLETEN-HOSOGLU & al. [21]). The salinity is also another factor affecting the growth and lipid formation in marine microalgal cultures (JIANG and CHEN [14]; BUMBAK & al. [22]). In the present study, CCD for three variables (yeast extract, glycerol and sea salt) along with their low, medium and high levels, was used as the second experimental design model for optimization of growth and lipid production of *C. cohnii*. According to the implemented design, twenty combinations were performed. In the experimental design model, sea salt (8-24 g L^{-1}), glycerol (10–30 g L^{-1}) and yeast extract (1.4–3.4 g L^{-1}) were taken as input variables. Cell dry weight (g L^{-1}) and the total lipid content were taken as responses of the system. A second order polynomial model obtained from the CCD for predicting cell dry weight and total lipid content were given in Eqs. (4)-(5), respectively:

$$\text{Cell dry weight (g L}^{-1}) = 7{,}51 - 0{,}44X_1 + 2{,}07\,X_2 + 0{,}33\,X_3 - 1{,}17X_2^2 - 0{,}88\,X_3^2 \quad (4)$$

$$\text{Total lipid content (\%w w}^{-1}) = 25{,}14 + 0{,}5X_1 + 2{,}03\,X_2 - 1{,}85X_3 - 1{,}57X_1^2 \quad (5)$$

where X_1, X_2 and X_3 are sea salt, glycerol and yeast extract, respectively.

The ANOVA of the regression model demonstrated that the model was highly significant, as indicated by the calculated F-value of 62.76 for cell dry weight, 9.49 for total lipid content. The values of Prob> F for cell dry weight (0.0001), total lipid content (0.0005) were all less than 0.01, indicating that the models for all responses were significant. According to the ANOVA test, the effects of sea salt, glycerol and yeast extract on cell dry weight were significant. As sea salt concentration in the culture medium increased, a decrease in cell dry weight was observed. A decrease in cell dry weight was also observed when the initial yeast extract concentration exceeding 2.4 g L^{-1}. In previous studies, yeast extract had been used in a wide range (1–11.5 g L^{-1}) and no limitation had been described before on growth of *C. cohnii* (DE SWAAF & al. [4; 5]; MENDES & al. [6]; DA SILVA & al. [12]). Pleissner and Eriksen [13] reported biomass productivity values between 0.72-1.0 g L^{-1} d^{-1} for *C. cohnii* in batch bioreactor productions. JIANG and CHEN [14] reported that, *C. cohnii* ATCC 30556 and *C. cohnii* ATCC 50051 strains achieved their highest biomass concentration (2.51 g L^{-1} and 1.56 g L^{-1}, respectively) at 9 g L^{-1} NaCl. The biomass productivity obtained in the current study was up to 1.0 g L^{-1} d^{-1}. The lower growth rate observed when the initial

glycerol concentration exceeded 20 g L^{-1} may probably be due to glycerol inhibition. In addition to that, there was no obvious change in cell dry weight when the initial glycerol concentrations exceeded 25 g L^{-1}. The yield coefficient of $Y_{X/S}$ on glycerol obtained in this study was between 0.30-0.48 (w w^{-1}). It was very close to the typical yield value obtained on glucose (0.45-0.50, w w^{-1}) (JIANG and CHEN [18]).

The final lipid content of flasks (20-30 %, w w^{-1}) obtained in optimized culture of *C. cohnii* in this study was significantly higher than those reported in previous researches (DE SWAAF & al. [4]; PLEISSNER and ERIKSEN [13]. According to the ANOVA test, effects of glycerol and yeast extract on total lipid content were significant. As the initial glycerol concentration in the culture medium increased, there was also an increase in total lipid content. On the contrary, as the yeast extract concentration increased, there was a decrease in total lipid content. This observation was in agreement with published data on other strain of *C. cohnii* (DE SWAAF & al. [4]. There was also an increase in total lipid content up to the sea salt concentration of 16 g L^{-1}. After that concentration, a decrease was observed in total lipid content. RATLEDGE & al. [7] tested the effect of independently varying the concentrations of sea salt and yeast extract in the growth medium of *C. cohnii*. They observed no significant improvement in lipid accumulation or DHA production by changes in concentrations of these nutrients. YEESANG and CHEIRSILP [23] reported that growth and lipid accumulation by newly isolated strains of *Botryococcus* spp. affected by salinity. In the present study, there was no significant difference between high salinity (29.45 g L^{-1}) and average salinity (16 g L^{-1}) in terms of both growth and lipid accumulation by *C. cohnii* which was in agree with DE SWAAF & al. [4]. Therefore, it was decided that the salinity of 16 g L^{-1} in culture medium was ideal for growth and lipid accumulation by *C. cohnii*. For large scale productions, it is also more preferable to use low sea salt concentrations because of corrosion problems (DE SWAAF & al. [4]). According to the results of experimental designs, the culture medium of *C. cohnii* was changed in a way for improved growth and lipid accumulation.

Nile red protocol should be developed for different algal species because of their different cell structure (ISLETEN-HOSOGLU & al. [21]; CHEN & al. [24]). In this current study, a detailed NR protocol was developed for screening of cellular neutral lipid content of *C. cohnii*. Effect of cell concentration on lipid quantification was investigated by mixing lyophilized powder of *C. cohnii* at different OD values of 0.05–0.400 using PBS solution. Before that, optical density value was transformed to cell number (*10^4 mL^{-1}) and the cell number was transformed to cell dry weight (g L^{-1}), by Eq. (1) and Eq. (2). A 2 mL algal suspension was sonicated for 15 s and then was mixed with the Nile red at final concentration of 0.2 μg mL^{-1}. Effect of cell concentration on the relative fluorescence intensity is depicted in Fig. 1(a). The fluorescence intensity of Nile red stained cell was linearly correlated with the cell concentration of OD 0.05–0.10. A cell density of OD 0.100 was therefore considered optimum for correct measurements of fluorescence intensity.

Eight samples with cell densities of OD 0.100, and different lipid concentrations were mixed with Nile red at final concentration of 0.2 μg mL^{-1}. As shown in Fig. 1(b), relative fluorescence intensity at 578 nm of the stained cells was correlated to intracellular lipid content determined by gravimetrically which can be expressed as;

Fluorescence Intensity (FI) = 3.52 Lipid content (%, w w^{-1}) + 4.3; R^2=0.96 (6)

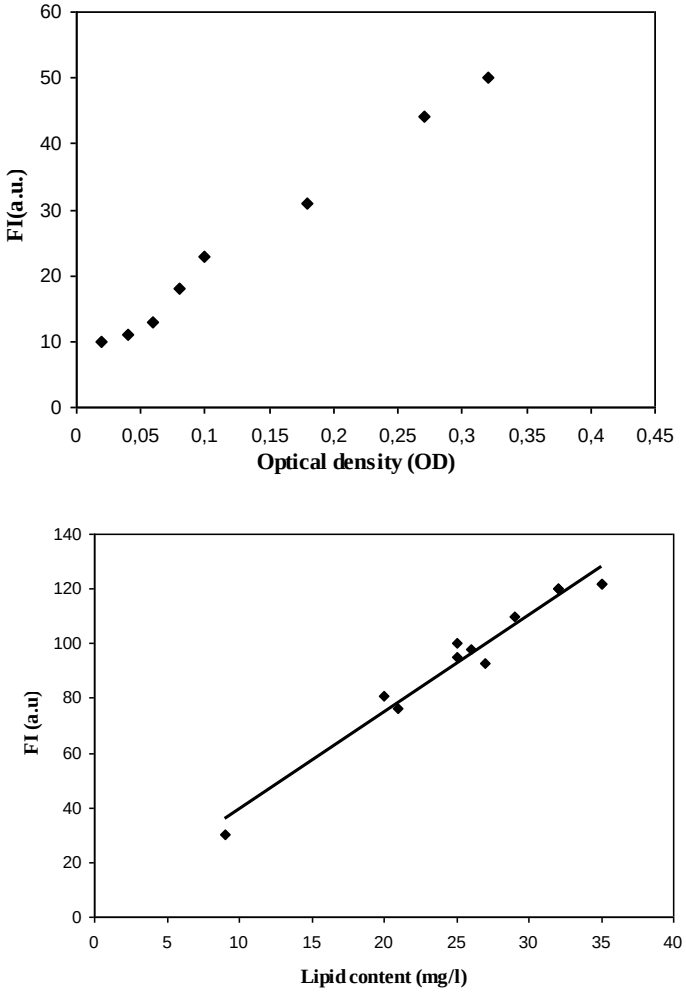
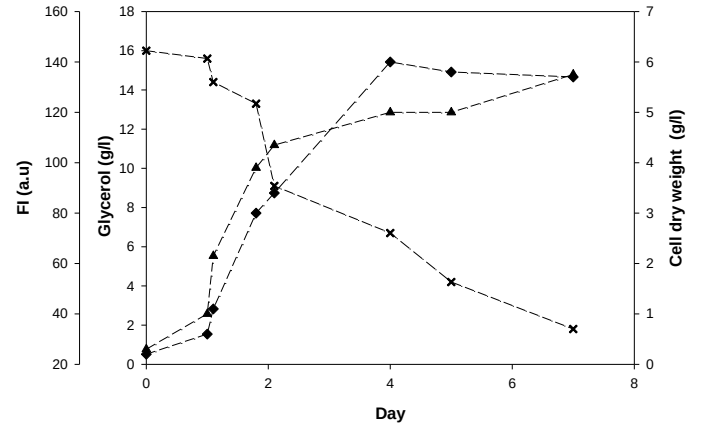


Fig. 1. (a) The effect of different concentrations (OD) of *C. cohnii* on the relative fluorescence intensity. Lyophilized alga powders were diluted to different concentration as OD 0.050–0.400 and mixed with 0.2 μg/mL Nile red, (b) the quantification of neutral lipid content of *C. cohnii* by relative fluorescence intensity ($p < 0.01$).

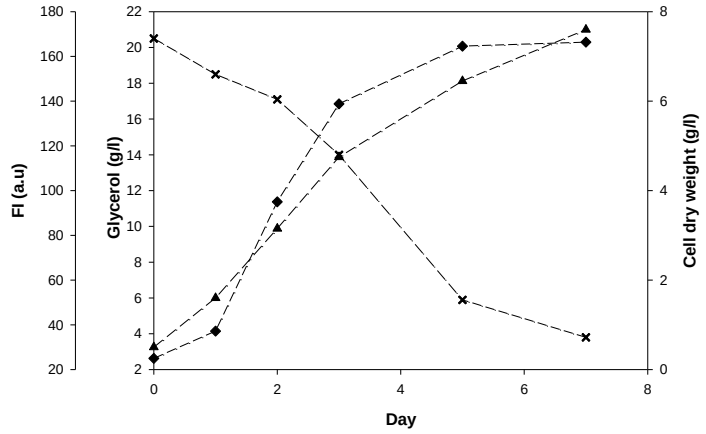
The main objective of this part was to investigate the effect of mechanical agitation on the growth and lipid production by *C. cohnii*. Bioreactor medium was consisted of optimized culture medium. The effect of three different mechanical agitation rates (100 rpm, 150 rpm and 250 rpm) on cell dry weight, glycerol consumption and lipid production due to fluorescence intensity were investigated. Also, the change in fatty acid composition during the growth was analyzed at optimum agitation rate.

The first bioreactor production was accomplished under 100 rpm agitation rate. After 7 days of cultivation, the cells entered the stationary phase, and the production ended up with a final cell dry weight around 5.7 g L^{-1} (Fig.2a). The initial glycerol concentration was around 16 g L^{-1} and not consumed by *C. cohnii* at the end of 7 days. The growth yield on glycerol was 0.46 g g^{-1}. The second bioreactor production was accomplished under 150 rpm agitation rate. After 7 days of cultivation, the cells entered the stationary phase, and the production ended up with a final cell dry weight around 7.3 g L^{-1} and a yield around 0.40 g g^{-1} on glycerol (Fig. 2b). And the last one was performed under 250 rpm agitation rate. After 8 days of cultivation, the cells entered the stationary

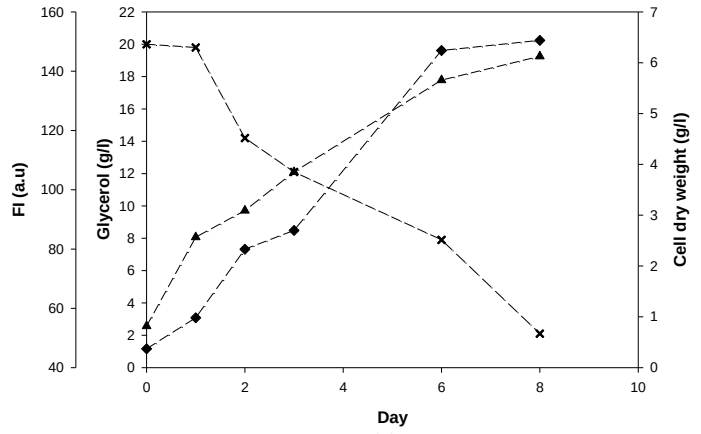
phase, and the production ended up with a final cell dry weight around 6.4 g L^{-1} and a yield around 0.36 g g^{-1} on glycerol (Fig. 2c).



(a)



(b)



(c)

Fig. 2. Growth (OD), glycerol consumption, lipid content as fluorescence intensity (FI) of *C. cohnii* in a 2 L stirred tank bioreactor at (a) 100 rpm, (b) 150 rpm, (c) 250 rpm stirrer rate. Cell dry weight (g/l, ♦), glucose consumption (g/L, **x**), flourescence intensity (a.u, ▲).

Nile red fluorescence intensity from stained cellular neutral lipids was measured starting from first day until the end of all productions. As seen in Figure 1, the lipid accumulation with time followed a similar trend to that of the cell growth for all bioreactor productions until the cells entered the stationary phase. Therefore, it could be said that lipid production was growth-associated. But, it also continued to increase after cells entered the stationary phase. Therefore, lipid production by *C. cohnii* differed from other microalgae species in which only growth-associated lipid accumulation were observed (ISLETEN-HOSOGLU & al. [21]; YANG & al. [25]). PLEISSNER and ERIKSEN [13] reported that changes in biochemical composition and cell morphology continued after nutrients were depleted, and also after the cultures entered stationary phase in batch cultures. RATLEDGE & al. [7] reported growth-associated lipid production for strain ATCC 30772. This developed method for Nile red based flourescence intensity is very useful especially for industrial scale allowing when to harvest microalgae for lipid production. The present study makes a significant contribution to this process with a developed method for quantification of lipid production by *C. cohnii* via flourescence measurement.

There were also differences in total lipid and DHA content between biomass. Total lipid content of biomass obtained from second bioreactor (36.5 %, w w^{-1}) was higher than that of the first bioreactor (28.0 %, w w^{-1}). On the other hand, there was no difference in total lipid content obtained from second and third bioreactor productions. When considering the biomass productivity and total lipid content for three bioreactor productions, the better results were obtained with the second bioreactor condition (150 rpm stirrer rate). Second bioreactor condition was also better in terms of the total specific DHA amount (49 mg g^{-1}). The total DHA amount obtained from first and third bioreactors were 34 mg g^{-1} and 32 mg g^{-1}, respectively. The third bioreactor production performed the lowest biomass productivity and DHA amount. With increasing aeration rates, cells had an enhanced supply of oxygen and multiplied more rapidly than cells grown with a restricted supply of oxygen (DE SWAAF & al. [4]). However, cell proliferation of dinofilagellates is negatively affected by mechanical agitation and can cause damage on microalgal cells (DA SILVA & al. [12]). Also, high mechanical agitation rates results in the power input increase. Generally, increasing stirrer speeds (between 100- 1000 rpm) were used to maintain a dissolved oxygen concentration between 30-40 % of air saturation for *C. cohnii* cultivation in bioreactors (DE SWAAF & al. [4]; RATLEDGE & al. [7]; MENDES & al. [11]). Therefore, it was important to find the optimum stirrer rate. Increasing agitation rate led to a decrease in biomass productivity and not affected the total lipid content by *C. cohnii* observed in this study. According to the results presented above, it was decided that 150 rpm agitation rate was better for *C. cohnii* cultivation when we considered all those factors. PLEISSNER and ERIKSEN [13] studied on batch cultures of *C. cohnii* grown on acetic acid and glucose using increasing stirrer speeds between 450-800 rpm to maintain a dissolved oxygen concentration above 40 % air saturation. They reported that total biomass amount obtained at the end of four days production was approximately 3 g L^{-1}; total lipid content was around % 15-23 (w w^{-1}); the specific DHA concentration was around 38 mg g^{-1}.

There were several researches on growth and lipid accumulation in *C. cohnii* by using glucose as the sole carbon substrate (DE SWAAF & al. [4]; JIANG and CHEN [14]. There was only one study mentioning the possibility of using glycerol for *C. cohnii* cultivation MENDES & al. [11]. In the present study, effect of using glycerol as

a carbon source was examined in terms of both growth and lipid accumulation by *C. cohnii*. A growth yield on glycerol was between 0.36-0.46 (w w^{-1}) for bioreactor productions. This value was very close to the yield on glucose JIANG and CHEN [18] and higher than other alternative carbon sources like ethanol (0.31 w w^{-1}) and acetic acid (0.12-0.22 w w^{-1}) (DE SWAAF & al. [5]; RATLEDGE & al. [7]; DE SWAAF & al. [8]. The technical aspects of commercial process of *C. cohnii* are obviously kept secret. However, it is estimated that glucose is used as a carbon source RATLEDGE & al. [7]. The cost of glucose is estimated to be about 80 % of the total medium cost. Therefore, the glucose in the medium might be replaceable LI & al. [26]. The results being reported here would indicate that an alternative process based on glycerol as the principal carbon source might seek to rival the traditional glucose process. By using glycerol, by-product of biodiesel production, it is also possible to cut down the production cost. The results obtained in this work indicated that glycerol could be potentially good carbon substrate for *C. cohnii* cultivation.

It is known that growth phase might affect fatty acid content and composition of microalgal cells (JIANG and CHEN [15]; FIDALGO & al. [27]). Therefore, the biomass collected during the second bioreactor production was analyzed to investigate the change in fatty acid content during the growth phase. The DHA proportion increased during the linear growth phase (3^{rd} day) until the beginning of stationary phase (5^{th} day). The maximum change in DHA proportion was observed on the 5^{th} day. There was a little change in DHA proportion during the stationary phase (7^{th} day). In addition to that, there was a decrease in C14:0 and C16:0 fatty acid proportions at the beginning of stationary phase (5^{th} day) and then an increase in following days (7^{th} day). Similarly, DE SWAAF & al. [4] stated that the percentage of DHA steadily increased as the culture aged up to the middle of stationary phase. On the other hand, they stated that the percentages of other fatty acids remained at a constant level or decreased slightly.

Statistical approaches employed in this study resulted in a better understanding of the nutritional requirements for optimum growth and lipid accumulation by *C. cohnii*. There was no need of using glutamic acid and sodium β-glycerophosphate in the basal medium of *C. cohnii* when yeast extract was used in the growth medium. Also, it was revealed that there was a reduced growth by *C. cohnii* with a certain yeast extract concentration. The sea salt concentration in the culture medium affected both growth and lipid production by *C. cohnii*. Therefore, optimum sea salt concentration was determined in this study. The results being reported here would also indicate that an alternative process based on glycerol as the principal carbon source was comparable to the traditional glucose process for *C. cohnii*. Effects of different agitation rates on biomass and lipid productivity and also specific DHA amount were determined in batch bioreactor cultivations. With the optimization of agitation rate in bioreactor, changes in fatty acid composition during the production were revealed.

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References

[1] Y. SHEN, W. YUAN, Z. PEI, E. MAO. Heterotrophic culture of *Chlorella protothecoides* in various nitrogen sources for lipid production. *Appl. Biochem. Biotechnol*, 160: 1674 (2010).

[2] O. PEREZ-GARCIA, F.M.E ESCALANTE, L.E. DE-BASHAN, Y. BASHAN. Heterotrophic cultures of microalgae: metabolism and potential products, *Water Res*, 45: 11 (2011).
[3] M. ISLETEN-HOSOGLU, D, AYYILDIZ-TAMIS, G. ZENGIN, M. ELIBOL. Enhanced growth and lipid accumulation by a new *Ettlia texensis* isolate under optimized photoheterotrophic condition. *Bioresour. Technol*, 131,258 (2013).
[4] M.E. DE SWAAF, T.C. DE RIJK, G. EGGINK, L. SIJTSMA. Optimisation of docosahexaenoic acid production in batch cultivation by *Crypthecodinium cohnii*. *J. Biotechnol*, 70, 185 (1999).
[5] M. E. DE SWAAF, L. SIJTSMA, J.T. PRONK. High-cell density fed-batch cultivation of the docosahexonoic acid producing marine alga *Crypthecodinium cohnii*. *Biotechnol. Bioeng*, 81, 666 (2003a).
[6] A. MENDES, P. GUERRA, V. MADEIRA, F. RUANO, T.L. DA SILVA, A. Reis, Study of docosahexaenoic acid production by the heterotrophic microalga *Crypthecodinium cohnii* CCMP316 using carob pulp as a promising carbon source. *World J Microbiol. Biotechnol*, 23, 1209 (2007).
[7] C. RATLEDGE, K. KANAGACHANDRAN, A.J. ANDERSON, D.J. GRANTHAM, J.C. STEPHENSON. Production of docosahexaenoic acid by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. Lipids, 36,1241 (2001).
[8] M.E DE SWAAF, J.T. PRONK, L. Sijtsma, Fed-batch cultivation of the docosahexaenoicacid-producing marine alga *Crypthecodinium cohnii* on ethanol. *Appl. Microbiol. Biotechnol*. 61, 40 (2003b).
[9] Z. CHI, D. PYLE, Z. WEN, C. FREAR, S. CHEN. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. *Process Biochem*., 42, 1537 (2007).
[10] J, O'GRADY, J.A. MORGAN. Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. *Bioprocess Biosyst. Eng*, 34, 121 (2010).
[11] A. MENDES, A, REIS, R. VASCONCELOS, P. GUERRA, T.L. DA SILVA. *Crypthecodinium cohnii* with emphasis on DHA production: a review. *J Appl. Phycol*, 21, 199 (2009).
[12] T.L. DA SILVA, A. MENDES, R.L. MENDES, V. CALADO, S.S. ALVES, J.M.T. VASCONCELOS, A. REIS. Effect of n-dodecane on *Crypthecodinium cohnii* fermentations and DHA production. *J Ind. Microbiol. Biotechnol*. 33, 408 (2006).
[13] D. PLEISSNER, N. ERIKSEN. Effects of Phosphorous, Nitrogen, and Carbon Limitation on Biomass Composition in Batch and Continuous Flow Cultures of the Heterotrophic Dinoflagellate *Crypthecodinium cohnii. Biotechnol. Bioeng*, 109, 2005 (2012).
[14] Y. JIANG, F. CHEN. Effects of salinity on cell growth and docosahexaenoic acid content of the heterotrophic marine microalga *Crypthecodinium cohnii*, *J Ind. Microbiol. Biotechnol*. 23, 508 (1999).
[15] Y. JIANG, F. CHEN. Effects of Temperature and Temperature Shift on Docosahexaenoic Acid Production by the Marine Microalga *Crypthecodinium cohnii*. JAOCS, 77, 613 (2000a).
[16] C.F. MANDENIUS, A. BRUNDIN. Bioprocess optimization using design-of experiments methodology. *Biotechnol. Prog*, 24, 1191 (2008).
[17] J. WANG, W. WAN. Experimental design methods for fermentative hydrogen production: A review. *Int. J. Hydrogen Energy*, 34, 235 (2009).
[18] Y. JIANG, F. CHEN. Effects of medium glucose concentration and pH on docosahexaenoic acid content of heterotrophic *Crypthecodinium cohnii*. Process Biochem, 35, 1205 (2000b).
[19] E.G. BLIGH, W.J. DYER. A Rapid Method of Total Lipid Extraction and Purification. *Canadian J. Biochem. Physiol*. 37, 911 (1959).
[20] W.W. Christie, in Lipid Analysis: Isolation, Separation and Structural Analysis of Lipids: Preparation of derivates of fatty acids, (Christie, W.W., ed.), J. Barnes and Associates, Dundee, Scotland, pp. 205–22 (2003).
[21] M. ISLETEN-HOSOGLU, I. GULTEPE, M. ELIBOL. Optimization of Carbon and Nitrogen Sources for Biomass and Lipid Production by *Chlorella saccharophila* under Heterotrophic Conditions and Development of Nile Red Fluorescence Based Method for Quantification of Its Neutral Lipid Content. *Biochem. Eng. J*. 61, 11 (2012).
[22] F. BUMBAK, S. COOK, V. ZACHLEDER, S. HAUSER, K. KOVAR. Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. *Appl. Microbiol. Biotechnol*. 91, 31 (2011).
[23] C. YEESANG, B. CHEIRSILP. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand, *Bioresource Technol*. 102, 3034 (2011).
[24] W. CHEN, C. ZHANG, L. SONG, M. SOMMERFELD, Q. Ç. HU, A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J. Microbiol. Methods* 77, 41 (2009).

[25] J. YANG, E. RASA, P. TANTAYOTAI, K.M. SCOW, H. YUAN, K.R. HRISTOVA. Mathematical model of *Chlorella minutissima* UTEX2341 growth and lipid production under photoheterotrophic fermentation conditions. *Bioresource Technol*. 102, 3077 (2011).
[26] X. LI, H. XU, Q. WU. Large-Scale Biodiesel Production From Microalga *Chlorella protothecoides* Through Heterotrophic Cultivation in Bioreactors. *Biotechnol. Bioeng.* 98, 764 (2007).
[27] J.P. FIDALGO, A. CID, E. TORRES, A. SUKENIK & C. HERRERO. Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga *Isochrysis galbana*. *Aquaculture*, 166, 105 (1998).