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# Synergistic Effects of pH, Temperature and Agitation on Growth Kinetics and Docosahexaenoic Acid Production of *C. cohnii* Cultured on Different Carbon Sources

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***Abstract* – *Crypthecodinium cohnii,* a heterotrophic marine dinoflagellate, has capability to produce docosahexaenoic acid (DHA) about 30–50 % of their total fatty acids. This property of *C. cohnii* to produce DHA as sole PUFA makes this alga to be an ideal candidate for industrial exploitation. Therefore present study was conducted to elucidate the effect of temperature, initial pH, agitation and different carbon sources on growth and cell biochemical composition especially DHA of *C. cohnii.* The results of individual variables revealed that highest biomass (15.1 g/L) was obtained when glycerol was used as carbon source at normal culture conditions. However highest lipid content (26.4 % DCW) was at temperature 20 °C; highest DHA content at acetic acid supplementation (43.9 % TFA); and the maximum overall lipid productivity (0.61 g/L.d) at glycerol supplementation. While, highest biomass content (21.9 g/L), lipid content (5.7 g/L) and DHA content (2.7 g/L) was achieved under synergy of temperature 20 °C, pH 6.5, aeration speed 300 rpm and glucose as carbon source. The use of glucose as major carbon and energy source would reduce the cost of industrial production of DHA from this alga.**

***Keywords* – *Crypthecodinium cohnii,* Docosahexaenoic Acid, DHA, LC-PUFAs, Temperature, Carbon Sources, Agitation.**

## I. INTRODUCTION

In recent years interest in long chain polyunsaturated fatty acids (LC-PUFA), especially omega-3 (n-3) and omega-6 (n-6) PUFA, has augmented greatly due to their diverse physiological functions in human health. Two important LC-PUFA belongs to omega-3: docosahexaenoic acid (DHA; 22:6n3) and eicosapentaenoic acid (EPA; 20:5n3), have been precisely recognized to involve in human nutrition, prevention of diseases, brain development, and particularly in infant formulae [1, 2]. The optimal concentration of these LC-PUFAs in the body is believed to maintain the normal functioning of cardiovascular, immune and anti-inflammatory system [2]. Moreover, higher concentration of PUFA especially DHA in the brain and retina is beneficial for nervous system and visual functions [3]. As human lack the enzymes involved in *de novo* synthesis of n-3 PUFAs, therefore, an external source is essential from the diet, especially for infants. Traditionally, fish oils have been considered the only major sources for industrial production of these LC-PUFA. Though, their use is limited due to poor oxidative stability, unpleasant taste, vulnerability to contaminations and a typical fishy smell [4]. Alternatively, several oleaginous microorganisms are now considered to be important sources of these important LC-PUFAs. Compared with other oleaginous microorganisms, microalgal DHA possesses various advantages, such as low EPA content, easy and cost effective separation and purification techniques [4].

Of key importance is *Crypthecodinium cohnii,* a heterotrophic marine dinoflagellate, have capability to produce DHA as sole LC-PUFA about 30–50 % of their total fatty acids. This property of *C. cohnii* to produce DHA as sole PUFA makes this alga to be an ideal candidate for industrial exploitation. For optimal DHA production final biomass content, total lipid and DHA content are important parameters [5]. Several strategies have been applied to improve lipid and PUFA production in microalgae. These include optimization of physical parameters (temperature, agitation, pH or light intensity), medium compositions (type and concentration of carbon source, nitrogen source, salts and other nutrients) and culture conditions (heterotrophic, phototrophic or mixotrophic) [6-8]. However, detailed information concerning DHA production from *C. cohnii* at different culture conditions is still limited. Only a fewer studies have reported the experimental quantification of lipids and DHA under the influence of temperature, pH, salinity and carbon sources in a limited number of *C. cohnii* strains [9-12]; whereas, in other microalgae, extensive studies have been carried out to elucidate the effect of different environmental factors on lipid content and fatty aid composition [13].

Additionally, the degree and direction of these factors on oleaginous microorganism are specie specific; as in some microalgae, these environmental conditions often have negative effect on growth and lipid content [14, 15], and so may result in different fatty acid productivity. For example, in some microorganisms especially diatoms, a rapid decline in EPA/DHA have been reported with culture age; in contrast, the EPA/DHA content increased with culture age in other microalgae [16]. Therefore, further studies are required on variations of the EPA/DHA content under different culture conditions. Besides, numerous studies reported the combined effect of different environmental conditions on lipid accumulation in diverse algal strains; no study has been done on *C. cohnii*. Therefore, present study was designed to enhance lipid and DHA production by synergistically optimization of different culture conditions. For this individual and combined effect of temperature, pH, agitation and different carbon source was evaluated on biomass, lipid and cell biochemical compositions of *C. cohnii*. Fatty acid shift was also appraised under differ culture conditions.

## II. METHODOLOGY

### *A. Microorganism and Growth Conditions*

*Crypthecodinium cohnii* strain (ATCC 30555) used in the present study was obtained from the America Type Culture Collection (ATCC) and maintained in sterilized ATCC460 medium for seven days. Cryovials were made up to a final 30% v/v glycerol concentration and stored at−80 °C. Starter cultures were prepared in 250 mL flasks with 100 mL working volume containing yeast extract (1.8 g/L) glucose (9 g/L) and sea salt (23 g/L) kept in static incubator at 25 °C for four days. Inocula were prepared in shake-flask cultures (150 rpm, 500 mL) containing 250 mL medium composed of yeast extract (2 g/L) glucose (27 g/L) and sea salt (25 g/L) inoculated with 10% v/v of static culture and grown at 27°C for 7 days. Batch cultures were performed in 1L Fermenters (NBS Bioflo 115, USA) with 800 mL of experimental medium. The optimal medium designed for algal growth without effecting lipid accumulation contains (g/L): sea salt 27 g/L, yeast extracts 5 g/L and glucose 25 g/L (for control). The optimal culture conditions were temperature 25 C and pH 6.5, Stepwise dissolved oxygen tension was achieved by shifting agitation speed from 700-300 rpm.

To evaluate the effect of different culture condition on the growth and DHA production; incubation temperature, initial pH, agitation speed and different carbon sources were examined on *C. cohnii*. For this a range of temperature (15 ºC to 40 ºC), pH (4 - 10), and agitation speed (150 - 450 rpm) were tested. Different carbon sources (sucrose, glactose, glucose, ethanol, acetic acid and glycerol) were used in this experiment to enhanced DHA production. All materials including medium in the fermenters was autoclaved at 121 °C for 20 min. Temperature was maintain at 27 ºC. All fine chemicals were purchased from Sigma-Aldrich unless otherwise stated and three replicates were performed in all experiments.

### *B. Determination of Physiological Parameters*

Dry cell weight (DCW, g/L) was gravimetrically measured from daily harvested samples by centrifugation (3000×g for 10 min, 5ºC). The cell pellet was rinsed twice with distilled water, frozen overnight at -80 ˚C and weighted following the lyophilisation for 24h. The specific growth rate ($\mu$, per day) was calculated using following equation (I)

$$\mu = \ln(DCW_f / DCW_i) / (T_f - T_i) \qquad (1)$$

Where, $DCW_f$ is the final biomass production (g/L); $T_f$ is the harvesting time (day); $DCW_i$ is the initial biomass production (g/L); $T_i$ is the cultivation time (day).

The biomass productivity ($P_{DCW}$) was calculated using following equation (II):

$$P_{DCW}(g/L.d) = (DCW_f - DCW_i) / (T_f - T_i) \qquad (II)$$

Glucose concentration was determined using glucose oxidase Perid-test kit (Shanghai Rongsheng Biotech Co., Ltd).

### *C. Total Lipids and Fatty Acid Profile*

Lipids were extracted by a modified protocol of [17] from freeze-dried cells. Lipid productivity ($P_{\text{Lipid}}$) and DHA productivity ($P_{\text{DHA}}$) were calculated by following formulae:

$$P_{Lipid}\,(g/L.d) = (C_f \times DCW_f - C_i \times DCW_i) / (T_f - T_i) \qquad (III)$$

$$P_{DHA}\,(g/L.d) = [C_{DHA}\,(g/g\ TL) \times Lipid(g/L)] / T \qquad (IV)$$

Where, $C_f$ is the final lipid content (g/L); $C_i$ is the initial lipid content; TL is total lipid.

For fatty acid analysis, ~50 mg of lyophilized algal biomass was re-suspended in 5 mL chloroform:methanol (2:1 v/v). 2.0 mg/mL pentadecanoic acid (C15:0) was added as an internal standard. 0.5 mg/mL Butylated hydroxytoluene (BHT) was added as an antioxidant This mixture was kept at room temperature for 24 h and then centrifuged (, 3000 × *g* for 5 min). The supernatant containing extracted lipids were transferred into a clean and washed with 2 mL of saturated NaCl. 2 mL hexane was added for extraction of fatty acid methyl esters (FAMEs) and analyzed by gas chromatography (GC-2010; Shimadzu Co., Kyoto, Japan). GC conditions were: capillary DB-WAX column (30 m × 0.32 mm, ð 0.25 µm, Agilent, USA), FID detector and helium as carrier gas. The oven temperature was initially held at 120 ºC for 3min and reached 180 ºC at 5 ºC per min, then raised to 260 ºC at 5 ºC per min, and finally held at 260 ºC for 5 min. The FAs were identified with standards (Sigma, USA).

### *D. Determination of Biochemical Composition*

Starch content was determined according to the available protocol of [18] with little modification made by [1]. Briefly, 50-60 mg of freeze dried biomass was resuspended in 4 mL of 20 mM tris buffer (pH 6.8) after washing twice. Algal cells were disrupted by ultra-sonication (225 bursts × 4 s; cooling amid) on ice for 15 min, and then centrifuged. The pellets including starch and cell debris were resuspended in 80% ethanol and incubated for 5 min at 85 ºC to remove glucose and maltodextrins. Subsequently, mixtures were centrifuged (8000 × *g*, 10 min, 4 ºC), resuspended in 2 mL DMSO containing thermo-stable α-amylase (Sigma, St. Louis, Mo, USA) and boiled for 5 min to digest the resistant starch. After cooling down, mixture was incubated in shaking water bath for 15 min by adding amyloglucosidase preparation (Sigma, St. Louis, Mo, USA). After final centrifugation (4000 × *g*, 15 min), starch content of the each sample was measured as equivalents to the glucose that was released to the supernatant. Total protein content was determined from biomass and culture medium by a modified version of Sun et al. 2017 [3]. Briefly, 25-50 mg of freeze dried DCW were resuspended in 1 ml of NaOH (0.5 M) and cell were disrupted by ultrasonically disruption (225 bursts of 455W lasting 4 s with cooling in between) using Scientz-II D sonifier (Ningbo Scientz Biotechnology Co., Ltd., China). Disrupted cells were incubated at 300 ºC for 24 h and centrifuged (15000 × *g,* 15 min). Total proteins in the supernatant were quantified using the bicinchoninic acid assay [19]. Bovine serum albumin was used as standard.

### *2.5 Statistical Methods*

Three biological replicates were performed in all experiments and analyzed by one-way analysis of variance (ANOVA) using SPSS 19. For optimal DHA production, different combinations of culture variables were subjected to orthogonal matrix design (OMD) with (pH, agitation, temperature and carbon source) at different levels (Table 1). Sixteen different experiments (run 1-16) were performed on the bases of OMD results of which were presented Table 2 in coded name.

## III. RESULTS AND DISCUSSION

To investigate the effects of different culture conditions on growth, lipid accumulation and DHA production, four different culture parameters (pH, Temperature, Agitation and carbon source) were studied independently and in combination for their synergetic effect. When independent culture condition was tested, all other culture parameters were kept constant similar to that of normal (control) culture conditions (see 2.1) in order to draw particular conclusions on the basis of respective variable effect on growth and lipid accumulation. For synergetic effect of tested parameters, coded values of independent variables by OMD is given in Table 1. All experiments were performed in 1 L fermenters with 800 ml working medium containing 25 g/L glucose, 5 g/L yeast extract, and 27 g/L sea salt.

### *A. Effect of Temperature*

The biomass, lipid and DHA content of *C. cohnii*, cultured on 25 g/L glucose as carbon source, 2 g/L yeast extract and 27 g/L sea salt, were tested at six different temperatures (15, 20, 25, 30, 35, 40 °C). After 7 day of cultivation, the biomass (DCW, g/L) was obtained at different temperatures are presented in Fig. 1a. It is obviously clear that algal growth varied in a significant manner at different temperatures. Biomass at temperature 25 - 30 °C was significantly higher (12.1 - 12.5 g/L) than at 15 and 40 °C. Biomass at temperatures 15 and 40 °C was almost similar and lowest (6.8 g/L and 5.2 g/L, respectively). The specific growth rate ($\mu$) was also lowest at these temperatures (0.52 $d^{-1}$). However, relatively higher growth rate up to 0.8 $d^{-1}$ was obtained at 25 and 30 °C (Fig. 1b). This indicated that lower temperature (15 °C) or higher temperature (40 °C) was not suitable for *C. cohnii* growth.

Temperature is considered as key factor for algal growth and other biochemical compositions. There was negative correlation between DHA content and cultivation temperature. Higher DHA content (34.9 - 36.4 % TFA) was obtained at 15 - 20 °C which was almost 40 % higher than that at 40 °C (Fig. 1a). Lipid content (% DCW) also decreased with increase in temperature (Fig. 1b). Highest lipid content (26.3 % DCW) was observed at 20 °C which was 60 % higher than that at 40 °C. In contrast, starch content increased with the increase in temperature and reached to a maximum value of 56 % DCW. No significant change was observed in protein content of the cell and a small but non-significant fluctuation from 13.4 – 14.6 % DCW was observed at all tested temperatures. As for as the biomass and lipid productivities are concerned (Fig. 1c), due to the fact that for lipid productivity amount of DCW gained is taken into account, a significant fluctuation was noted ($p > 0.05$).

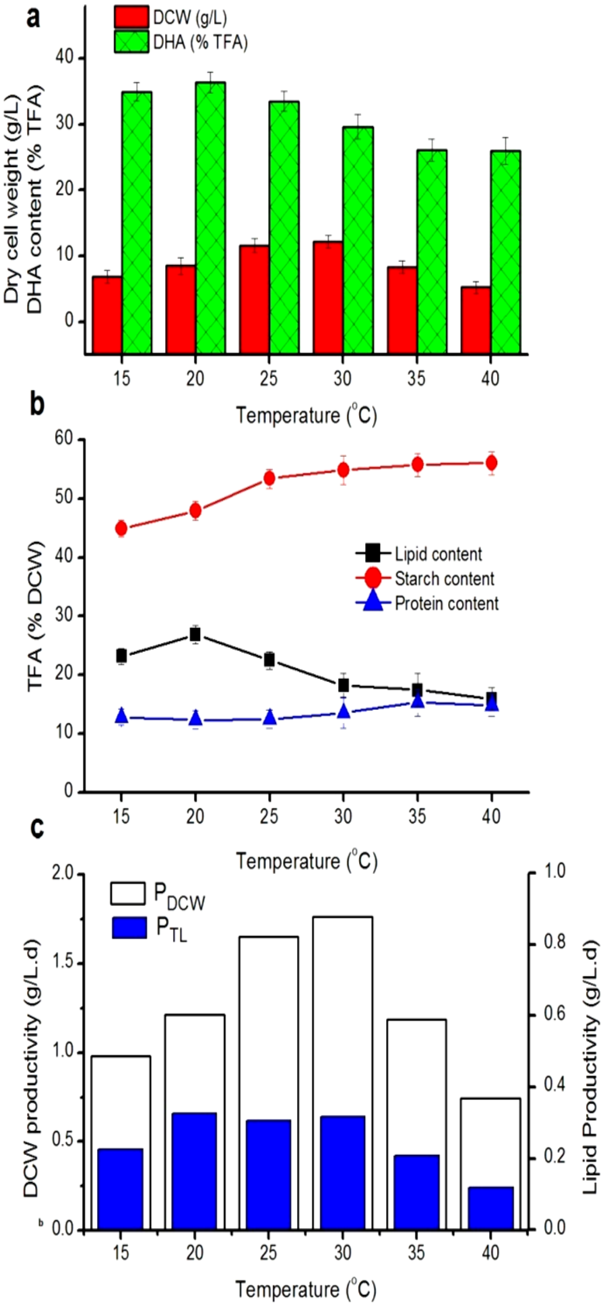

Fig. 1. Effect of temperature on **(a)** dry cell weight (DCW, g/L) and DHA content (% TFA), **(b)** cell biochemical composition (lipid, starch, protein; % of DCW), **(c)** biomass productivity ($P_{DCW}$, g/L.d) and lipid productivity ($P_{TL}$, g/L.d) of *C. cohnii* after 7 days of cultivation. Values shown are the mean of three independent experiments ± standard deviation.

These results indicated that the optimal temperature for growth and overall lipid and DHA productivity was 25 – 30 °C. Both maximum biomass productivity (1.8 g/L.d) and lipid productivity (0.32 g/L.d) were obtained at 30 °C. Our results are in accordance with the other studies on C. cohnii, that optimal temperature for growth and lipid production is between 25 – 30 °C [1,3,9-12] with highest results at 27 °C [10]. The optimal temperature for other algal strains was also between 25 – 30 °C; for example, 27 °C for *Chlorella vulgaris* [20, 21], 28 for *Chlorella* sp. HQ [22], 25°C for *S.*

*limacinum* [23] and 25 for *Chlamydomonas reinhardtii* [24].

### B. Effect of Initial Culture pH

In the present study *C. cohnii* was able to grow on a wide range of pH from 5.0-9.0 (Fig. 2a). However, the pH values lower than 5.0 or higher than 9.0 were found to be unfavorable for *C. cohnii* growth as growth was completely ceased after 24 h of cultivation. Moreover, at pH 3 and 11, *C. cohnii* growth was completely ceased even for a cultivation period of 10 days in the shake flask. Although, *C. cohnii* grow on a wide range of pH, regardless of that, a similar growth pattern was observed in three growth stages as described in our previous work. However, increased growth was observed from pH 4.0 to 6.5 and then declined at higher pH (7.0-9.0). The maximum biomass content (DCW, 12.5 ± 1.8 g/L) was obtained from the culture at initial pH 6.5. The optimal pH range for *C. cohnii* was 6.0-7.0 with highest biomass yield at pH 6.5 (Fig. 2a). These results are in accordance with the finding of Kyle et al. (1991) [25]. In contrast, Jiang and Chen (2000) reported highest growth yield of *C. cohnii* at pH 7.2 when cultured on glucose as major carbon source [26]. The optimal pH range for other microalgae *Chlorella vulgaris* was 5.0-9.0 and beyond this pH lysis of the cells was observed within the first two days of cultivation [27].

pH is an important environmental factor have influence on cell surface properties and alters permeability of ions, acids and bases across the membrane. pH also affect cell biochemical metabolism including lipids carbohydrate and proteins. However, nutrient depletion such as nitrogen, phosphorus or sulfur also influences cell biochemical events in microorganism; therefore, in the present study access supple of nutrients was supplied. As shown in Fig. 2b, with the increase of pH, lipid content (% DCW) also increased slightly. More specifically, higher lipid content was obtained between pH 6.0-7.0 with highest value at pH 6.5 of 22.4 ± 2.5 % DCW. DHA production was slightly affected by initial pH and highest DHA content (43.6 % TFA) was obtained at pH 6.5 (Fig 2a). In contrast, saturated fatty acids (C14:0, C16:0, C18:0) and unsaturated fatty acids (C16:1, C18:1, C18:2) were remained unchanged in all medium pH. In *C. cohnii*, glycolipids of photosynthetic membranes are not present and DHA is present as sole polyunsaturated fatty acid (PUFA) in the form of TAG [28]. There is no significant change in lipid and DHA content at pH 5.0 - 7.0.

The comparison of dry cell weight and lipid productivities (g/L.d) offers a better understanding of algal growth at different initial medium pH. Fig. 2c presents the biomass and lipid productivities; complementing with the growth, highest biomass productivity ($P_{DCW}$, 1.8 g/L.d) and lipid productivity ($P_{TL}$, 0.4 g/L.d) was achieved at pH 6.5. While, lowest $P_{DCW}$ and $P_{TL}$ was obtained beyond pH 5 and 9 which is less than 0.02 g/L.d (Fig. 2c). It is reported that intracellular pH of alga is maintained by initiation of Na$^+$/proton and K$_+$/proton antiport systems along with primary cellular proton pump [28]. This creates a transmembrane electrical potential with positive charge inside the cell at the expense of cellular energy. Beyond the optimal pH for growth of *C. cohnii,* more energy is required to stabilize the intracellular pH so that cell can perform its biochemical functions normally [9]. Therefore, biomass content and productivities decreased at pH lower then 5 and higher than 9.

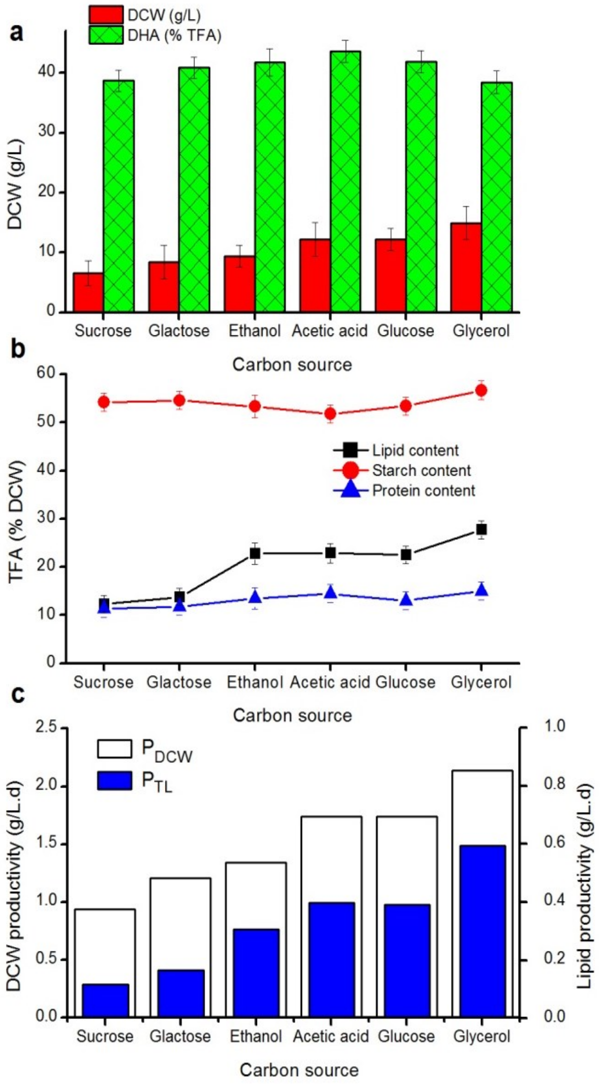

Fig. 2. Effect of pH on **(a)** dry cell weight (DCW, g/L) and DHA content (% TFA), **(b)** cell biochemical composition (lipid, starch, protein; % of DCW), **(c)** biomass productivity ($P_{DCW}$, g/L.d) and lipid productivity ($P_{TL}$, g/L.d) of *C. cohnii* after 7 days of cultivation. Values shown are the mean of three independent experiments ± standard deviation.

### C. Effect of Agitation

When all nutrients were present in the medium in sufficient amount and optimum culture conditions were adopted, the specific growth rate only depends on the availability of dissolved oxygen. Biomass, lipid and DHA content and productivities are shown in Fig. 3. There was a slight change in the biomass and DHA content with changing agitation speed from 150 to 450 rpm (Fig 3a). However no significant difference was observed in cell biochemical composition (lipid, starch, protein) at all tested agitation speeds (Fig 3b). A slight but significant increase was observed in overall biomass and lipid productivity when agitation speed was increased from 150-450 rpm (Fig

3c). It is reported that at higher aeration conditions, oxygen supply play an important role in growth and productivities; cells multiply more rapidly at high supply of oxygen as compared to limited $O_2$ supply [29]. When agitation speed was increased from 150 - 300 rpm, specific growth rate inc2reased to the highest value (0.71 $d^{-1}$), however, increase in agitation speed above 300 rpm reduced the growth rate to 0.66 $d^{-1}$. The microalga *P. cruentum* was sensitive to excessive agitation and growth rate decreased above 350 rpm [30].

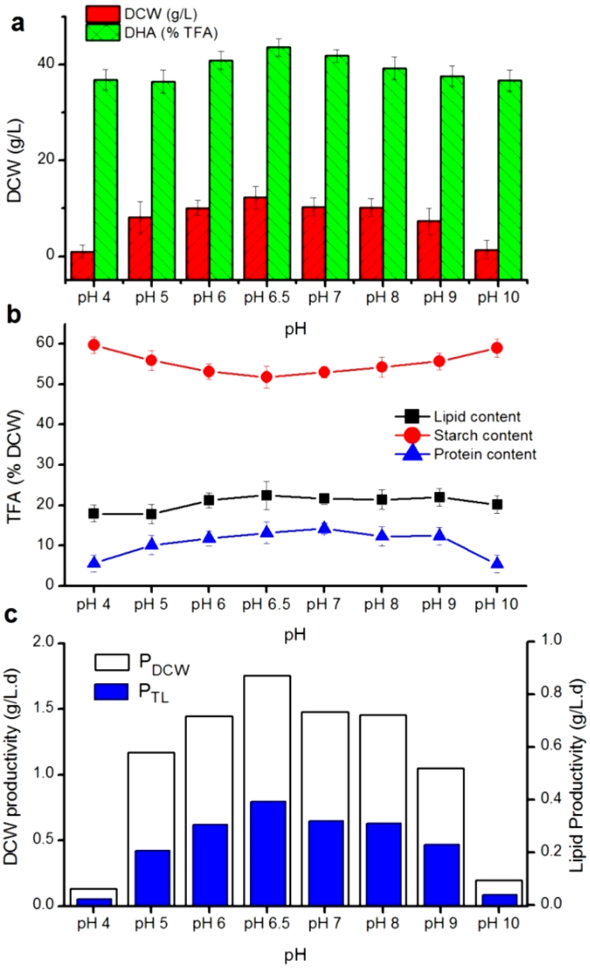

Fig. 3. Effect of agitation on **(a)** dry cell weight (DCW, g/L) and DHA content (% TFA), **(b)** cell biochemical composition (lipid, starch, protein; % of DCW), **(c)** biomass productivity ($P_{DCW}$, g/L.d) and lipid productivity ($P_{TL}$, g/L.d) of *C. cohnii* after 7 days of cultivation. Values shown are the mean of three independent experiments ± standard deviation.

### *D. Effect of Carbon Sources*

Carbon generally play the most important role in fermentation because this nutrient is directly associated with biomass and cell metabolites. In order to determine a suitable carbon source for growth and lipid especially DHA production, six different carbon sources were tested. Traditionally glucose was used as carbon source for *C. cohnii* cultures; the optimal growth rate and biomass was achieved at 20-25 g/L glucose among the tested 4 – 40 g/L [9]. In another study acetic acid, glucose, ethanol, D-fructose and glycerol were favorable for optimal growth and DHA production in *C. cohnii* [31]. Glycerol and ethanol when used as carbon source elicited DHA productivity comparable to that seen for glucose however highest DHA content was obtained with glycerol [31].

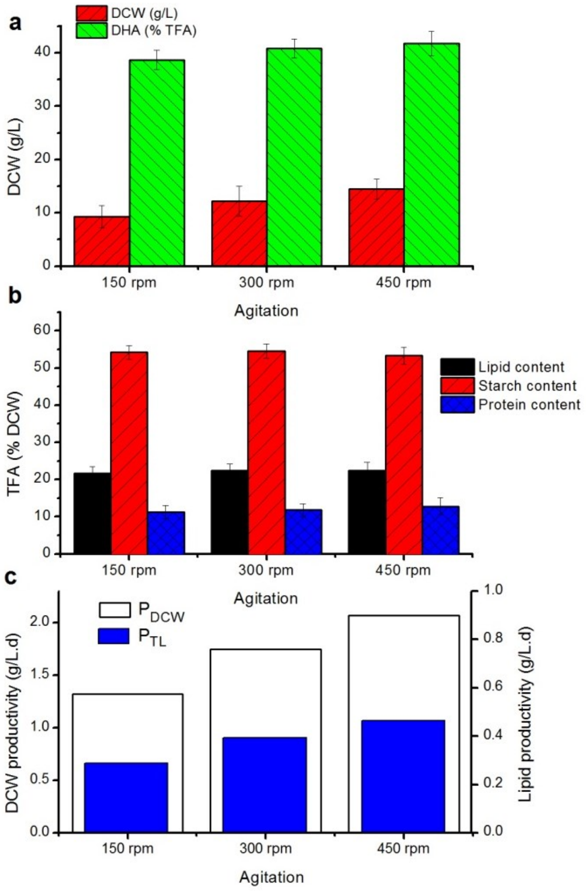

Fig. 4. Effect of carbon source on **(a)** dry cell weight (DCW, g/L) and DHA content (% TFA), **(b)** cell biochemical composition (lipid, starch, protein; % of DCW), **(c)** biomass productivity ($P_{DCW}$, g/L.d) and lipid productivity ($P_{TL}$, g/L.d) of *C. cohnii* after 7 days of cultivation. Values shown are the mean of three independent experiments ± standard deviation.

In the present study, among the tested carbon sources, highest biomass was obtained with glycerol (15.1 g/L) and acetic acid (12.4 g/L) which is comparable to glucose (12.6 g/L) (Fig. 4a). Lowest biomass yield was obtained with sucrose and glactose which was 50-90% lower than that of glucose. Nevertheless, no significant change was observed in DHA content in all tested C-sources, though highest DHA content was obtained with acetic acid (Fig. 4a). Specific growth rate (Fig. 4a) in ethanol, acetic acid and glucose was comparable (0.64-0.68 $d^{-1}$) which is however 6-10 % lower than glycerol (0.72 $d^{-1}$). Biochemical compositions of the cell were also comparable in all glucose supplementation (Fig. 4b). There was a significant difference in biomass and lipid productivities and highest value biomass and lipid production was attained with glycerol; acetic acid and glucose showed almost similar results (Fig. 4c). These results indicated that glucose acetic acid and glycerol are the most favorable carbon sources for *C. cohnii*. Glucose was used as major carbon and energy source in most of the commercial cultivations because it is easily accessible feedstock, water-soluble and cost effect then other sources [4].

Conclusively, among all individual variable in this experiment, highest biomass (15.1 g/L) was obtained when glycerol was used as C-source at normal culture conditions. However highest lipid content (26.4 % DCW) was at temperature 20 °C; highest DHA content at acetic acid supplementation (43.9 % TFA); and the maximum overall lipid productivity (0.61 g/L.d) at glycerol supplementation was obtained. Because of these diverse results at different culture conditions, synergetic effect of these tested culture conditions in random combinations on the basis of orthogonal matrix design was also carried out.

### *E. Synergetic effect of Culture Conditions*

The coded values of four independent variables (temperature, pH, aeration, carbon source) on the basis of orthogonal design (by SPSS 19) are presented in Table 1. The highest biomass (g/L) and lipid content (% DCW) was observed in assay 16, 10 and to some extent 15. Whereas, lipid content reached to its highest value of 27.7 % DCW with the assay 15, a little higher than assay 16; Assay 3 and 4 also give higher lipid content (22-23% DCW). Similarly, highest lipid content (5.7 g/L), DHA content (2.7 g/L), lipid productivity (816 mg/L.d) and DHA productivity (283 g/L.d) was obtained with assay 16 (Table 2). So it can be concluded that assay 16 in which conditions are (temperature 25 °C; pH 6.5; agitation speed 300 rpm; glucose as carbon source) were encountered the highest results among all 1-16 assays (Table 2). One of the prime objectives of this study was to enhance DHA content (% TFA) and yield (g/L). In *C. cohnii*, DHA is the sole PUFA in high amount more than 25-35 % of TFA [4], we successfully produce it in higher amount (46.9 % TFA) with a net yield of 2.7 g/L which is 2-4 fold higher than previous efforts [1,3, 9-12 ].

Table 1. Orthogonal matrix design for studied culture conditions (coded values for factors and their levels).

| Factors | Code 1 | Code 2 | Code 3 | Code 4 |
|---|---|---|---|---|
| Temperature | 15 ºC | 25 ºC | 30 ºC | 40 ºC |
| pH | 5 | 6.5 | 8 | -- |
| Carbon source | Sucrose | Ethanol | Glucose | Glycerol |
| Agitation | static | 150 rpm | 300 | -- |

Table 2. Orthogonal matrix design of synergy in different culture conditions (coded values) and responses in biomass content (DCW, g/L) lipid content (TFA, % DCW), DHA content (DHA, % TFA), lipid productivity ($P_{TL}$, mg/L.d) and DHA productivity ($P_{DHA}$, mg/L.d) after 7 days of cultivation. All experiments were performed in triplicate. The data presented here is mean ± standard deviation.

| Assay | pH | Agitation | Temperature | Carbon source | DCW | TFA | DHA | $C_{TL}$ | $C_{DHA}$ | $P_{TL}$ | $P_{DHA}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 4.3 | 11.5 | 36.9 | 0.5 | 0.2 | 70.6 | 26.1 |
| 2 | 3 | 3 | 1 | 3 | 10.5 | 20.7 | 39.1 | 2.2 | 0.8 | 310.5 | 121.4 |
| 3 | 1 | 1 | 1 | 4 | 7.2 | 23.8 | 36.9 | 1.7 | 0.6 | 244.8 | 90.3 |
| 4 | 2 | 2 | 1 | 2 | 8.5 | 22.5 | 38.2 | 1.9 | 0.7 | 273.2 | 104.4 |
| 5 | 2 | 1 | 3 | 1 | 4.5 | 13.8 | 38.5 | 0.6 | 0.2 | 88.7 | 34.2 |
| 6 | 1 | 3 | 2 | 1 | 6.8 | 12.6 | 38.2 | 0.9 | 0.3 | 122.4 | 46.8 |
| 7 | 3 | 2 | 4 | 1 | 4.2 | 13.9 | 37.5 | 0.6 | 0.2 | 83.4 | 31.3 |
| 8 | 1 | 1 | 4 | 3 | 3.6 | 12.9 | 25.5 | 0.5 | 0.1 | 66.3 | 16.9 |
| 9 | 1 | 1 | 4 | 2 | 2.8 | 12.5 | 25.2 | 0.4 | 0.1 | 50.0 | 12.6 |
| 10 | 1 | 3 | 3 | 2 | 7.9 | 18.5 | 38.5 | 1.5 | 0.6 | 208.8 | 80.4 |
| 11 | 2 | 3 | 4 | 4 | 5.9 | 17.5 | 46.2 | 1.0 | 0.5 | 147.5 | 68.1 |
| 12 | 2 | 1 | 2 | 3 | 5.5 | 25.9 | 36.1 | 1.4 | 0.5 | 203.5 | 73.5 |
| 13 | 3 | 1 | 3 | 4 | 5.1 | 18.9 | 25.8 | 1.0 | 0.2 | 137.7 | 35.5 |
| 14 | 3 | 1 | 2 | 2 | 4.6 | 15.9 | 23.9 | 0.7 | 0.2 | 104.5 | 25.0 |
| 15 | 1 | 2 | 3 | 3 | 16.9 | 27.7 | 44.9 | 4.7 | 2.1 | 668.8 | 300.3 |
| 16 | 2 | 3 | 2 | 3 | 21.9 | 26.1 | 46.9 | 5.7 | 2.7 | 816.6 | 383.0 |

$C_{TL}$: lipid content (g/L); $C_{DHA}$: DHA content (g/L); $P_{TL}$: lipid productivity (g/L.d); $P_{DHA}$: DHA productivity (g/L.d); $Y_L$All experiments were performed in triplicate. The data presented here is mean.

*C. cohnii* cultivated at low temperatures (15 - 20 °C) in combination with other variables; the fatty acid profile was 38.5 - 39.5 % saturated fatty acids, 54 - 64 % unsaturated fatty acids containing 36 - 46 % DHA as major component. *C. cohnii* cultured at high temperature (30 - 40 °C); the fatty acid profile was 32 - 39 % saturated fatty acids, 54 - 55 % unsaturated fatty acids containing 36 - 41 % DHA as major component. It was observed that C. cohnii produce more unsaturated fatty acids than saturated fatty acids and especially DHA upto 46 % of DCW. Previously it was reported that fatty acids in *C. cohnii* are composed of C14:0, C16:0, C18:0, C18:1, and DHA (C22:6), with 36.97% of TFA is DHA [32]. In this study sesamol was used to enhance DHA content, however, C18:1 significantly decreased to 8.5% with sesamol 1.5 mM treatment; DHA content significantly increased by 11.25% than the control cultures and reached to the highest value 41.13% of TFA [32].

Table 3. Fatty acid profile (% total fatty acid) of *C. cohnii* under different temperature, carbon source, pH and agitation speed on the bases of orthogonal matrix design. Samples were harvested on 120h of cultivation. All experiments were performed in triplicate. The data presented here is means.

| Assay | C14:0 | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | C22:6 | Others |
|---|---|---|---|---|---|---|---|---|
| 1 | 14.4 | 17.2 | 2.8 | 5.9 | 8.9 | 6.5 | 36.9 | 7.5 |
| 2 | 13.5 | 18.1 | 2.4 | 5.4 | 8.5 | 6.5 | 39.1 | 6.6 |
| 3 | 14.1 | 18.4 | 2.7 | 5.8 | 8.8 | 6.7 | 36.9 | 6.7 |
| 4 | 13.7 | 18.7 | 2.8 | 5.8 | 8.2 | 6.1 | 38.2 | 6.6 |
| 5 | 14.4 | 19.2 | 2.6 | 6.2 | 9.3 | 5.9 | 38.5 | 4.0 |
| 6 | 14.1 | 18.1 | 2.8 | 5.6 | 8.7 | 6.5 | 38.2 | 6.1 |
| 7 | 13.4 | 17.2 | 2.6 | 5.9 | 8.6 | 6.2 | 37.5 | 8.7 |
| 8 | 14.0 | 18.3 | 3.2 | 5.8 | 8.9 | 6.6 | 25.5 | 17.8 |
| 9 | 14.2 | 18.7 | 3.0 | 5.9 | 8.2 | 5.9 | 25.2 | 19.0 |
| 10 | 14.3 | 17.2 | 2.8 | 5.9 | 8.2 | 6.7 | 38.5 | 6.5 |
| 11 | 14.6 | 17.8 | 3.0 | 6.2 | 8.5 | 5.8 | 40.2 | 4.0 |
| 12 | 14.0 | 17.2 | 3.1 | 5.0 | 9.3 | 6.1 | 36.1 | 9.3 |
| 13 | 14.4 | 18.2 | 2.8 | 5.8 | 8.4 | 8.3 | 25.8 | 16.4 |
| 14 | 13.0 | 17.0 | 3.0 | 5.9 | 8.9 | 6.1 | 21.9 | 24.3 |
| 15 | 13.9 | 17.5 | 2.5 | 5.9 | 8.5 | 6.2 | 41.9 | 3.7 |
| 16 | 13.3 | 18.3 | 3.3 | 5.4 | 8.5 | 6.5 | 42.9 | 1.9 |

## CONCLUSION

The ability of C. cohnii to produce DHA could be enhanced by selecting proper culture conditions and medium compositions. Highest biomass content (21.9 g/L), lipid content (5.7 g/L) and DHA content (2.7 g/L) was achieved under synergy of temperature 20 °C, pH 6.5, aeration speed 300 rpm and glucose as carbon source. The use of glucose as major carbon and energy source would reduce the cost of industrial production of DHA from this alga. However, there is still much room to enhance DHA production in *C. cohnii* by using different strategies. Conclusively, this study could be useful in further exploitation of industrial DHA production from *C. cohnii*

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