

Synergistic Effect of Phosphorus and Nitrogen on Growth, Lipid Accumulation and Docosahexaenoic Acid Production in *Cryptocodinium Cohnii*

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Abstract — Docosahexaenoic acid (DHA) has numerous health benefits and widely used as an important component of infant formulae due to its prominent role in fetal brain and retina development. In this study, the synergetic effects of phosphate and nitrate concentrations on growth, lipid especially DHA biosynthesis and cell biochemical composition have been investigated in *Cryptocodinium cohnii*. The highest biomass was attained at synergy of high nitrogen (N) and phosphate (P, 23.5 ± 3.7g/L). When P concentration was increased from 0.1 g/L to 0.5 g/L with HN supplementation, biomass also increase by approx. 22%, however, at 2.0 g/L of P biomass decreased by 20 % which was unpredictable. Though, at higher P concentration, glucose dissimilation rate was higher (620 mg/L.h) as compared to at lower P concentration (330 mg/L.h). On the other hand, maximum TFA (36 % DCW) were produced at low P (0.1 g/L) concentration which was 35-40 % higher than that of high P (2.0 g/L) concentration. The maximum DHA content (3.3 g/L) and DHA productivity (0.54 g/L.d) was also obtained with 0.1 g/L KH₂PO₄. In this study, no significant effect was observed on lipid accumulation under LN or HN supply. It indicated that nitrogen limitation is not a trigger for lipid accumulation in *C. cohnii*. Therefore, a comprehensive study is required to evaluate this different mechanism of lipid accumulation.

Keywords — *Cryptocodinium Cohnii*, Docosahexaenoic Acid, DHA, LC-Pufas, Nitrogen Limitation, Phosphate, Lipid Accumulation.

I. INTRODUCTION

Docosahexaenoic acid (DHA, C₂₂:6n₃) is one of the most important long chain polyunsaturated fatty acids (LC-PUFAs) have garnered increasing attention worldwide over the past decade. DHA has numerous beneficial effects on animals and human health, including improvement of neural development, visual acuity, hypertension; reduction of cardiovascular diseases, arthritis and arteriosclerosis [1]. Moreover, DHA is considered as an important component of infant formulae and other infant related food products due to their prominent role in fetal brain and retina development [2]. Previously, fish oils have been used as major source for LC-PUFAs; however, their use is limited due to poor oxidative stability, unpleasant taste, vulnerability to contaminations and a typical fishy smell [3]. Alternatively, several marine microorganisms including oleaginous fungi and microalgae are now considered to be the major 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].

Among numerous microalgal strains, *Cryptocodinium cohnii*, a heterotrophic marine dinoflagellate, have capability to produce DHA as single 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. Comparing to other oleaginous microalgae such as *Monoraphidium* sp., *Skeletonema costatum* and *Botryococcus braunii*, which can produce lipids more than 60% of their biomass [5], lipid production in *C. cohnii* is still relatively low. Many efforts have been made to elevate lipid production in *C. cohnii* with a maximum lipid content upto 20-25% of biomass with DHA accounting for 30-35% of the total lipid content [1, 3, 4, 6, 7, 8]. However, *C. cohnii* still have much room for lipid and DHA enhancement. As, DHA in *C. cohnii* is mainly present in the form of triacylglycerol (TAG), however, glycolipids (GL), phospholipids (PL) and Neutral lipids (NL), are the three main lipid classes [9]. Under adverse growth conditions most of the oleaginous microorganisms produce lipids in the form of TAG. Therefore, two-step cultivation process was usually carried out for higher TAG production. First, sufficient nutrients are supplied for optimal growth and then nutrient deficient conditions were applied to trigger TAG accumulation. However, mechanism of lipid accumulation was remained unexplained in these kinds of processes but improved lipid content can be achieved [8-9].

C. cohnii may also accumulate starch up to 40% of the biomass at the expense of same carbon and energy sources as that of lipids. So, inhibition of starch synthesis may lead to boost lipid accumulation; a strategy used to enhance lipid accumulation in *Chlamydomonas reinhardtii* [11]. In this study lipid content was significantly elevated by 3.5 folds by decreasing starch content under nutrient limitation as compared to nutrient sufficient conditions. However, this coordination of starch and lipid biosynthesis in *C. cohnii* has never been thoroughly studied till date. Therefore, it is also important to evaluate relationship between starch and lipid accumulation.

For optimal DHA production in *C. cohnii*, culture conditions, growth rate, final biomass concentration and total lipid content are important parameters. Previously, numerous studies have been carried out to optimize DHA production in *C. cohnii* by optimizing nutrients especially glucose, used as the carbon and energy source; represents an easily accessible feedstock for many industrial

fermentation processes [3]. It has been reported that nutrient limitations simultaneous with excess carbon source caused an unbalanced growth conditions which are believed to be favorable for lipid accumulation in most of the oleaginous microorganisms [10]. It was reported that *C. cohnii* can be grown in batch, fed-batch, and pH auxostat cultures on different carbon sources including glucose acetic acid, or ethanol. This indicates that *C. cohnii* supported cost effective lipid production by using inexpensive nutrients as their substrate and can be favorable for industrial production of DHA [12]. Therefore, glucose was used in the present study as major carbon source. Nitrogen (N) is regarded as the most important nutrient for increased lipid production when present in limited amount. However, high lipid production is directly associated with reduced cell proliferation and hence lower overall lipid productivity under nitrogen limitation. Phosphorus is also one of the essential elements for microorganisms and phosphate limitation strategy is commonly used to promote growth and lipid production.

Phosphate (P) is essential for cellular energy transfer; signal transduction and most importantly nitrate absorption. Phosphate assimilated by microalgae is stored in the form of polyphosphate granules (poly-P) and can be utilized when medium become phosphate deficient [13]. A few studies have been reporting the synergistic utilization of nitrogen and phosphorus to for improved lipid production in different microalgae [14]. However, no study has been done on *C. cohnii*. Therefore, present study was aimed to enhance lipid and DHA production by synergistically optimization of nitrogen and phosphate. For that, individual and combined effect of nutrient (N, P) limitation and supplementation was evaluated on biomass, lipid and cell biochemical compositions. Lastly, fatty acid shift was also appraised under differ nitrogen and phosphate treatments.

II. METHODOLOGY

A. Algal strain and growth conditions

Cryptocodinium cohnii strain (ATCC 30555) used in the present study was obtained from the America Type Culture Collection (ATCC). *C. cohnii* was cultured in sterilized ATCC460 medium for seven days from cryovial stocks and used to inoculate 800 mL of experimental medium in 1 L Fermenters (NBS Bioflo 115, USA) supplemented with low nitrogen source (LN, 0.1 g/L NaNO₃) or high nitrogen source (HN, 1.0 g/L NaNO₃). The optimal medium (pH 6.5) designed for algal growth without effecting lipid accumulation contains (g/L): Na₂SO₄, 3.9; KBr, 0.1; NaCl, 23.5; MgCl₂ · 6H₂O, NaHCO₃, 0.2; 10.6; CaCl₂, 1.2; KCl, 0.6; ZnCl₂, 0.1; glucose, 30; glutamic acid, 0.15; SrCl₂·6H₂O, 0.04; 5.0 mL of metal mixture (g/L): (Na₂EDTA, 10; MnCl₂·4H₂O, 1.6; H₃BO₃, 10; FeCl₃·6H₂O, 0.5; CoCl₂·6H₂O, 0.005); 3.0 mL of tris buffer; 1.0 mL of vitamin solution (mg/L): (biotin, 3; thiamine, 1000). Different concentrations of KH₂PO₄ (0.0, 0.1, 0.3, 0.5, 1.0, 2.0 g/L) was used as phosphate source in this experiment. All materials including medium in the fermenters was autoclaved at 121

°C for 20 min. Temperature was maintain at 27 °C. 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, 5°C). The cell pellet was rinsed twice with distilled water, frozen overnight at -80 °C and weighted following the lyophilisation for 24h. The biomass productivity (P_{DCW}) was calculated using following equation (1):

$$P_{DCW}(g/L.d) = (DCW_f - DCW_i) / (T_f - T_i) \quad (1)$$

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).

Glucose concentration was determined using glucose oxidase Perid-test kit (Shanghai Rongsheng Biotech Co., Ltd). Soluble phosphates were determined using colorimetric method [10] with little modification. The absorbance of the supernatant was measured at 885 nm, after proper dilution with deionized water. Phosphate concentration was determined by using a calibration curve made with KH₂PO₄ in the range 10-100 μM. Nitrate concentration in the medium was determined by spectrophotometer [15]. For that, daily harvested samples were centrifuged (3000 × g, 5°C for 10 min) and absorbance was measured from supernatant at 220 nm after proper dilution with deionized water [16]. The absorbance values were converted to nitrate concentration using a standard calibration curve made with NaNO₃ in the range 0-10 mM.

C. Total lipids and fatty acid profile

Lipids were extracted by a modified protocol of [16] from freeze-dried cells. Lipid productivity (P_{Lipid}) and DHA productivity (P_{DHA}) were calculated by following formulae:

$$P_{Lipid}(g/L.d) = (C_f \times DCW_f - C_i \times DCW_i) / (T_f - T_i) \quad (2)$$

$$P_{DHA}(g/L.d) = [C_{DHA} (g/g TL) \times Lipid(g/L)] / T \quad (3)$$

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, δ 0.25 μm, Agilent, USA), FID detector and helium as carrier gas.

The oven temperature was initially held at 120°C for 3min and reached 180 °C at 5 °C per min, then raised to 260°C at 5°C per min, and finally held at 260°C 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 [17] with little modification made by [1]. Briefly, 50-60 mg of freeze dried biomass was resuspended in 4mL of 20mM tris buffer (pH 6.8) after washing twice. Algal cells were disrupted by ultrasonication (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 °C to remove glucose and maltodextrins. Subsequently, mixtures were centrifuged (8000 × g, 10 min, 4 °C), 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 using Kjeldahl method [18] by Kjeldahl nitrogen-to-protein conversion factor. Three biological replicates were performed in all experiments and analyzed using a one-way analysis of variance (ANOVA) using SPSS Statistics 19.

III. RESULTS AND DISCUSSIONS

A. Synergistic effects of N and P on growth and substrates consumption

Phosphorus (P) and nitrogen (N) are important macronutrients for growth, metabolism and other cellular functions such as protein synthesis, ATP generation, enzyme activities and signal transductions [19]. In this work, the synergistic effects of different concentrations of P and N supplementation were investigated on growth, lipid accumulation particularly DHA and other cellular components on *Cryptocodinium cohnii* (Fig. 1). *C. cohnii* can grow on any concentration of phosphate (0 - 2 g/L); however, with decrease in P and nitrate concentration in the culture medium, growth of *C. cohnii* also decreased. When P concentration was increased from 0.1 g/L to 0.5 g/L, biomass also increase by approx. 22%, however, at 2.0 g/L of P biomass decreased by 20 % which was unpredictable. This behavior of alga is different from the normal trend of increase in biomass concentration with the increase in phosphate concentration [20, 21]. Whereas, slightly but significantly lower biomass was obtained when cultured without P or at low P concentrations as compared to high P supply. Simultaneously, time course profile analysis of growth showed that *C. cohnii* grow faster in the first 48 h of cultivation and then gradually decreases till end of cultivation time and entered in to stationary phase after 120 h. While in synergy with P and N starvation culture, the cell culture approached stationary

phase quickly after 48 h (Fig. 1). During first 48 h of cultivation, no significant difference was observed in growth under Low (LN) and high (HN) nitrogen conditions ($p > 0.05$); however, after 48 h cell proliferation ceased in LN condition (Fig. 1). A significantly ($p < 0.001$) higher biomass (DCW) was attained at HN concentration (18-23 g/L) as compared to LN concentration (9-11 g/L).

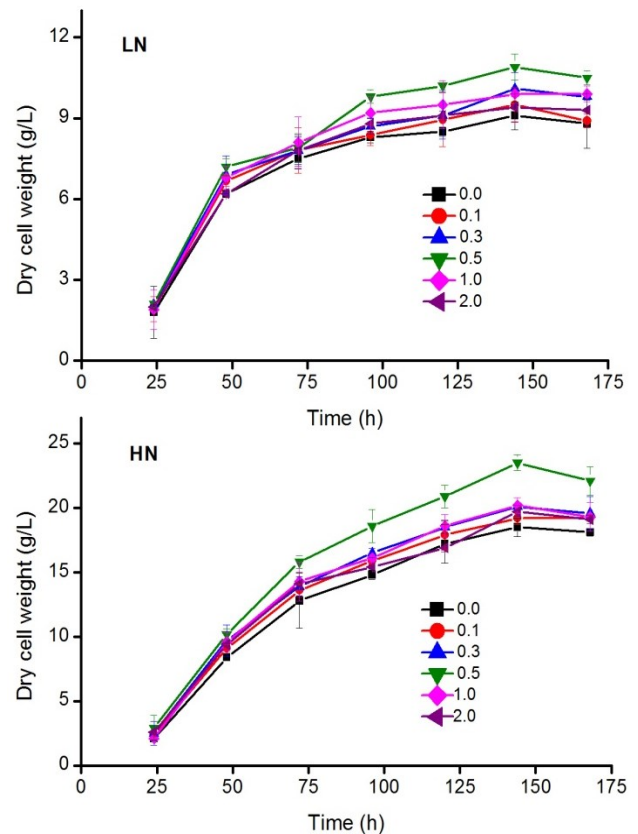


Fig. 1: Effect of different phosphate (KH_2PO_4) concentrations on biomass (DCW, g/L) content of *C. cohnii* under low (LN, 0.1 g/L NaNO_3) and high nitrogen (HN, 1.0 g/L NaNO_3) supplementation. Values shown are the mean of three independent experiments \pm standard deviation.

Glucose assimilation rate in *C. cohnii* is directly associated with growth; complimenting the growth, glucose assimilation rate gradually decrease during the cultivation time. It was observed that at LN treatment, glucose was dissimilated at a similar rate in all P treatments (400-40 mg/L.h). Nevertheless, dissimilation rate of glucose in HN supply was directly depends upon P treatment. At higher P concentration, glucose dissimilation rate was higher (620 mg/L.h) as compared to at lower P concentration (330 mg/L.h). These results suggested that synergetic effect of high N and P can induce higher growth in *C. cohnii*. Similar results were found when *Scenedesmus abundans* grown on high N and P supply [20]. Belotti et al., 2013 also reported lower biomass of *C. vulgaris* under low N and P supply [21].

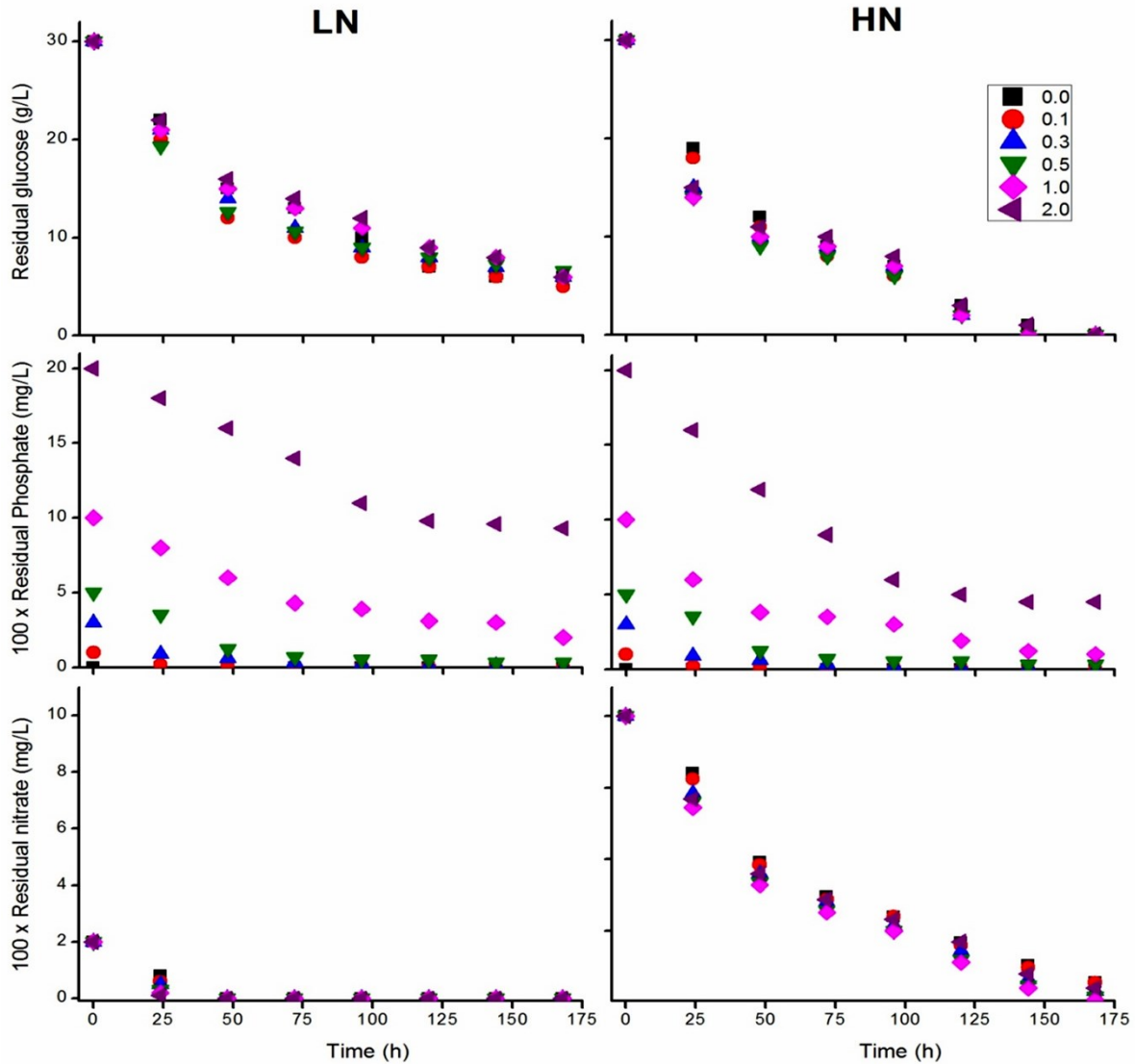


Fig. 2: Effect of different phosphate (KH_2PO_4) concentrations on residual assimilation (glucose, phosphate and nitrate) in *C. cohnii* cultured under low (LN, 0.1 g/L NaNO_3) and high nitrogen (HN, 1.0 g/L NaNO_3) for 7 days. All experiments were performed in triplicate. The data presented here is mean.

Biomass yield per gram glucose utilized (g/g Glc) was also calculated (Table 1). Highest biomass yield was obtained with 0.5 g/L K_2HPO_4 in combination with HN supply (0.78 ± 0.12 g/gGlc) which was 63 % higher than that from LN supply. During the batch cultivation, *C. cohnii* rapidly assimilated nitrate (NO_3^-) and (PO_4^{3-}) throughout the cultivation time (Fig. 2). Under LN supplementation, initial N concentration completed utilized within first 24 h and cells became N-starved thereafter. Conversely, N somehow remains available in the medium in HN supplementation till end of the culture time. On the other hand, P started to assimilate immediately after inoculations at higher rate 16 mg/L.h. Despite of the N availability, P continued to assimilate at a comparable rate in all treatments however lower supply of P exhausted earlier as compared to higher P supply. These results indicate that phosphate consumption is independent of N consumption.

Table 1: Combined effect of nitrogen and phosphate concentration on biomass yield (Y_{DCW} , mg/g GLC), lipid

yield (Y_{TL} , mg/g GLC) and DHA yield (Y_{DHA} , mg/g GLC) of *C. Cohnii*.

N-conc.	Phosphate Conc.	Y_{DCW}	Y_{TL}	Y_{DHA}
LN	0.0	379.2	127.0	64.8
	0.1	395.8	126.7	65.9
	0.3	439.1	123.0	56.6
	0.5	480.2	139.3	61.3
	1.0	450.0	121.1	50.8
	2.0	427.3	104.7	45.0
HN	0.0	637.9	209.9	104.7
	0.1	640.0	214.4	110.4
	0.3	670.0	200.3	88.9
	0.5	783.3	238.9	107.5
	1.0	673.3	175.7	73.8
	2.0	679.3	168.5	65.7

Yield was calculated on basis of glucose (g/L) utilized LN, low nitrogen, 0.1 g/L NaNO_3 ; HN, high nitrogen, 1.0 g/L NaNO_3 ; All experiments were performed in triplicate. The data

presented here is mean.

B. Synergistic effects of N and P on lipid and fatty acid composition

N and P not only influenced cell growth but also have significant effect on lipids accumulation and fatty acid composition in *C. cohnii*. As shown in Fig. 3a, regardless of LN or HN lipid commence to accumulate and reached a maximum at 120 h of cultivation (30-35% of DCW). Numerous studies have been reporting that nitrogen limitation is trigger to induce lipid biosynthesis in oleaginous microorganisms [21-24]. In this study, no significant effect was observed in lipid accumulation

under LN or HN supply. Lipids starts to accumulate in *C. cohnii* even N was still present in the medium. It indicated that triggers of lipid accumulation in *C. cohnii* are different from other oleaginous microorganism. Therefore, a comprehensive study is required to evaluate possible environmental stressed to enhance lipids for industrial production. The highest biomass was attained under HN and 1.0 g/L P concentration ($23.5 \pm 3.7\text{g/L}$) while maximum TFA (36 % DCW) were produced at 0.1 g/L P concentration which was 35-40 % higher than that of 2.0 g/L P conc. (Fig. 3a).

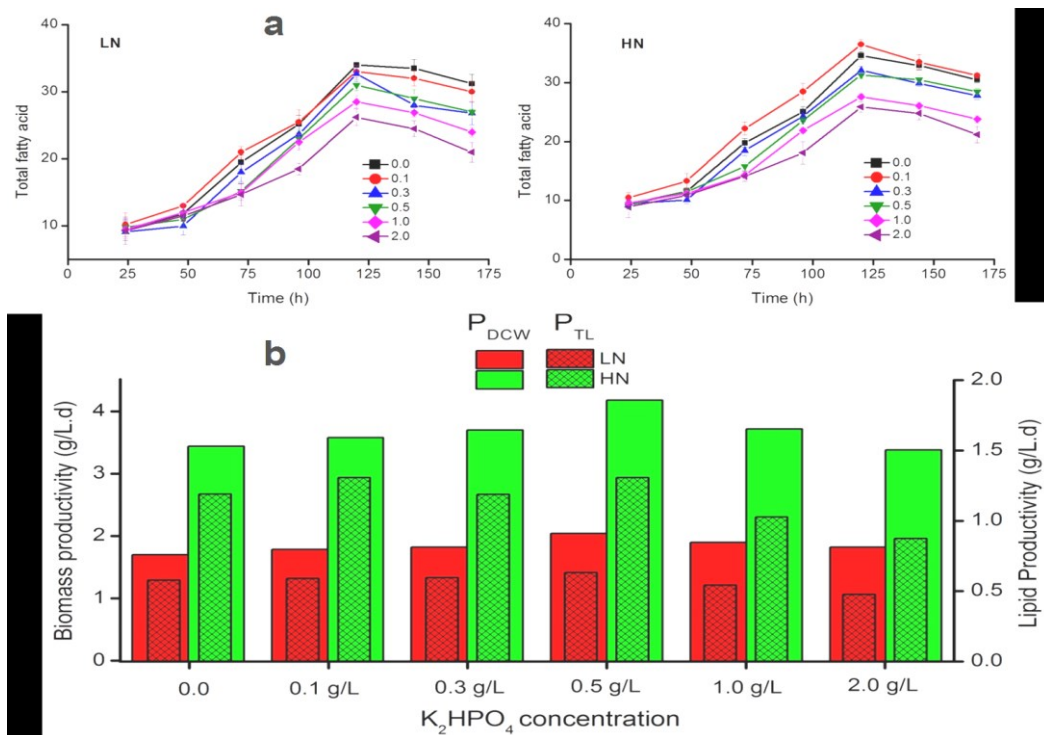


Fig. 3: Effect of different phosphate (KH_2PO_4) concentrations on (a) total fatty acid content (TFA, % DCW), (b) biomass productivity (P_{DCW} , g/L.d) and lipid productivity (P_{TL} , g/L.d) of *C. cohnii* under low (LN, 0.1 g/L NaNO_3) and high nitrogen (HN, 1.0 g/L NaNO_3) supplementations. Values shown are the mean of three independent experiments \pm standard deviation.

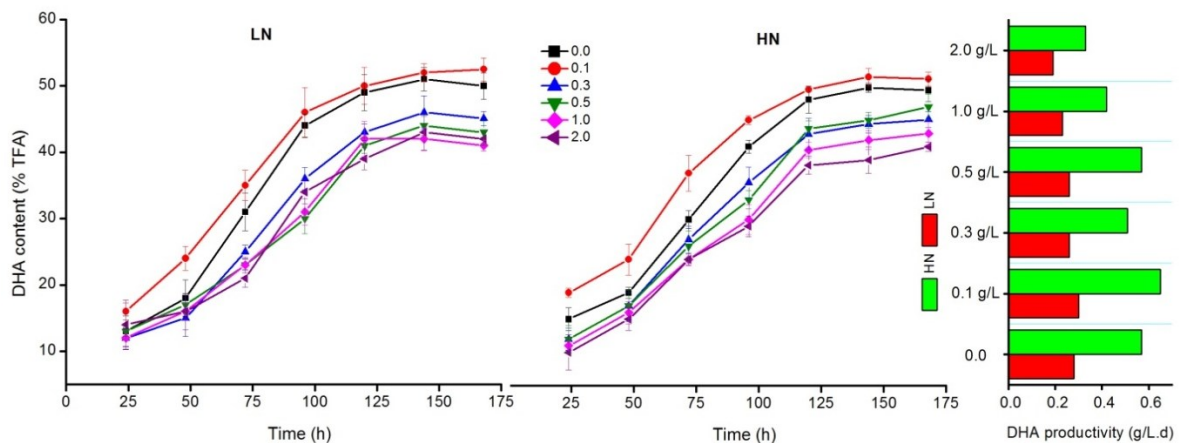


Fig. 4: Effect of different phosphate (KH_2PO_4) concentrations on (a) DHA content (% TFA) and DHA productivity (P_{DHA} , g/L.d) of *C. cohnii* under low (LN, 0.1 g/L NaNO_3) and high nitrogen (HN, 1.0 g/L NaNO_3) supplementations. Values shown are the mean of three independent experiments \pm standard deviation

Fig. 3b shows synergetic influence of P and N on biomass and lipid productivity. The maximum biomass productivity (4.1 g/L.d) and lipid productivity (0.9 g/L.d) was obtained at 0.5 g/L P treatment under HN. These results confirm the synergy of P and N on growth and lipid production in *C. cohnii*. Similar trend have been reported by Chu et al., 2014 in *Scenedesmus obliquus* and Chu et al., 2013 in *C. vulgaris* grown in phosphate limited condition [25-26]. Previously, it was reported that N deprivation in microalgae leads to cell proliferation due to protein degradation and release N which is utilized for the cellular metabolic processes in old cell [27]. While, P deficiency of the cells is fulfilled by utilization of the storage poly-P granules; accumulated during high P availability [28]. Our results suggested that P limitation was the main 'lipid trigger' while nitrogen limitation have no role in lipid accumulation. Highest lipid yield (g/g Glc) was obtained at 0.5 g/L P with HN treatment (238.9 ± 13.5 g/g Glc) which was 79% higher as compared to LN treatment (139.3 ± 9.2 g/g Glc) (Table 1). Conclusively, the maximum biomass and lipid productivity was achieved by culturing *C. cohnii* on 0.5 g/L K_2HPO_4 and 1.0 g/L $NaNO_3$. Therefore, for further analysis of cell biochemical composition, LP as 0.1 g/L K_2HPO_4 and HP as 0.5 g/L K_2HPO_4 was used in combination with LN and HN.

C. Fatty acid profile and cell biochemical composition

Table 2 showed the fatty acid shift in response to different combinations of N and P treatments. As one of the primary objectives of this study was to enhance DHA content; combined effect of P and N treatments was also elucidated on DHA content, DHA productivity (Fig. 4) and DHA yield (Table 1). Complimenting with lipid accumulation, N supplementation have no significant effect on DHA content (% of TFA). Time course profile showed a gradual increase in DHA throughout the cultivation time and reached to a maximum value of 52.1 ± 2.8 % TFA at 144 h of cultivation. Furthermore, reducing KH_2PO_4 concentration from 2.0 g/L to 0.1 g/L

increased DHA proportion by 30% indicating that low phosphorus source is favorable for DHA production. In contrast with lipid production, highest DHA productivity (0.54 g/L.d) and DHA yield (0.11 g/g Glc) was achieved by 0.1 g/L KH_2PO_4 . Other fatty acids accumulated by *C. cohnii* were at their maximum value at high P concentration which was 5-20% higher than that of low P treatments. Therefore, it could be concluded that for higher DHA production, low P concentration culture were favorable for *C. cohnii*.

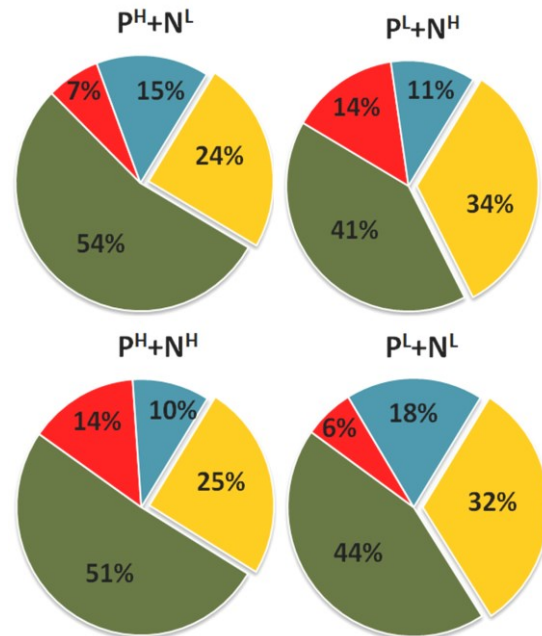


Fig. 5: Combined effect of different concentrations phosphate (KH_2PO_4) and nitrogen on total lipid (yellow), starch (green), protein (red) and other cell biochemical compositions (blue). Values shown are the mean of three independent experiments \pm standard deviation

Table 2: Fatty acid profile (% total fatty acid) of *C. Cohnii* under different phosphate concentrations at low (LN, 0.1 g/L $NaNO_3$) and high nitrogen (HN, 1.0 g/L $NaNO_3$) supplementations supplementation. Samples were harvested on 120h of cultivation. All experiments were performed in triplicate. The data presented here is means \pm SD.

N-conc.	KH_2PO_4 conc. (g/L)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	DHA	Others
LN	0.0	11.3 \pm 1.1	12.7 \pm 0.3	11.9 \pm 0.1	2.5 \pm 0.2	4.6 \pm 0.3	1.1 \pm 0.2	49.4 \pm 0.1	6.9 \pm 0.2
	0.1	9.8 \pm 1.3	12.0 \pm 0.9	12.5 \pm 0.1	1.7 \pm 0.1	7.2 \pm 0.9	2.4 \pm 0.4	50.2 \pm 1.2	4.5 \pm 0.7
	0.3	12.5 \pm 0.5	13.7 \pm 1.1	13.1 \pm 0.1	0.9 \pm 0.0	4.8 \pm 1.1	3.7 \pm 0.1	43.8 \pm 0.9	8.3 \pm 0.2
	0.5	14.9 \pm 0.3	19.1 \pm 0.3	9.4 \pm 0.1	2.6 \pm 0.3	5.6 \pm 0.3	0.9 \pm 0.1	41.1 \pm 1.2	6.5 \pm 1.3
	1.0	14.5 \pm 0.2	11.5 \pm 0.5	5.9 \pm 0.0	1.6 \pm 0.2	4.6 \pm 0.5	8.9 \pm 0.6	42.2 \pm 1.0	11.0 \pm 1.9
	2.0	15.5 \pm 0.5	13.2 \pm 1.3	6.9 \pm 0.1	4.9 \pm 0.1	9.5 \pm 1.3	3.2 \pm 0.1	39.4 \pm 1.3	7.8 \pm 1.2
HN	0.0	10.9 \pm 0.4	12.2 \pm 0.2	2.6 \pm 0.0	2.4 \pm 0.1	4.6 \pm 0.2	6.2 \pm 0.2	48.1 \pm 0.4	13.0 \pm 0.9
	0.1	9.9 \pm 0.7	11.5 \pm 0.3	9.8 \pm 0.5	1.2 \pm 0.3	8.3 \pm 0.2	0.6 \pm 0.1	49.6 \pm 0.9	9.2 \pm 0.8
	0.3	11.4 \pm 0.4	13.2 \pm 0.5	8.2 \pm 0.1	0.6 \pm 0.3	6.6 \pm 0.5	1.3 \pm 0.4	42.9 \pm 1.1	15.8 \pm 1.5
	0.5	14.8 \pm 0.3	15.4 \pm 0.3	5.8 \pm 0.0	5.2 \pm 0.6	3.6 \pm 0.3	0.9 \pm 0.1	43.7 \pm 0.3	10.6 \pm 1.3
	1.0	14.1 \pm 0.2	11.6 \pm 0.5	6.3 \pm 0.4	6.4 \pm 0.5	8.4 \pm 0.5	4.9 \pm 0.0	40.5 \pm 0.2	7.8 \pm 1.0
	2.0	15.9 \pm 0.5	10.2 \pm 1.3	9.5 \pm 0.1	3.5 \pm 0.1	5.7 \pm 1.3	7.2 \pm 0.1	38.2 \pm 0.3	9.8 \pm 1.2

LN, low nitrogen, 0.1 g/L $NaNO_3$; HN, high nitrogen, 1.0 g/L $NaNO_3$

All experiments were performed in triplicate. The data presented here is mean \pm standard deviation.

Starch is the major carbon and energy storage component of microorganisms accumulate at the cost of available carbon source in the medium. Hence, lipid and starch biosynthesis compete for carbon source. Therefore, to enhance lipids and DHA production, metabolic relationship between starch and lipids is important [1]. In contrast, with lipid accumulation, higher starch content was achieved at higher P supplementation (Fig. 5). However, N has no significant effect on starch content ($p < 0.05$). On contrary, protein content of the cell was significantly influenced by N supply. It was observed that under LN concentration protein content reduced to the half as compared to the HN supplementation. Recently, it is reported that protein content decreased in *C. reinhardtii* when grown in LN and LP as compared to HN containing media [30]. This is the first report on changes in cell biochemical content (especially starch, protein) grown in different N and P concentrations. Therefore, this study provides useful insights on cell biochemical changes during higher lipid production under selected stress conditions.

IV. CONCLUSION

Highest biomass content (23.5 g/L) and productivity (3.9 g/L.d) was achieved under synergy of high N and P. The overall highest lipid productivity (0.9 g/L.d) was attained at High N and P combination while, maximum DHA productivity (0.4 g/L.d) at high N and low P concentration. Starch content was also decreased by decreasing P concentration. Protein content was decreased to half under LN treatment as compared to HN treatment. Conclusively, synergistic limitation of N and P was an effective strategy to induce high lipid production at low consumption of substrate in *C. cohnii*.

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