

Responses in Growth and Lipid Productivity of *Chlorella Vulgaris* to Different Nitrogen Sources

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Abstract

Background: Nutrient compositions of the growth medium, nitrogen in particular, influence the microalgal biomass and lipid productivity.

Materials and Methods: *Chlorella vulgaris* was cultivated under varying nitrogen sources in the form of ammonium acetate (NH₄-N), calcium nitrate (NO₃-N), glycine, sodium nitrite (NO₂-N) and urea to improve biomass and lipid productivity. Specific growth rate, dry cell weight, cellular pigments, biomass concentration were taken as measurement of cell growth and lipid productivity was determined at the end of cultivation period.

Results: NO₃-N was demonstrated to be the best nitrogen source for both biomass and lipid-producing potential of *C. vulgaris* with 0.34 g L⁻¹ day and 0.126 g L⁻¹ day, respectively. The other nitrogen sources contributed to algal lipid-producing potential were NO₂-N and NH₄-N.

Conclusion: The findings suggests that the identification of appropriate N source provides an economically feasible strategy to obtain biomass and lipid productivity from microalgae simultaneously.

Keywords: Microalgae; *Chlorella Vulgaris*; Nitrogen; Nitrate; Biomass; Lipid

Introduction

Physiological composition of growth medium produces biochemical changes in microalgae which is also species-specific. In other words, change in the nutrient environment not only affect photosynthesis and growth rate of microalgae but also result in increase or decrease of cellular macromolecular composition [1-3]. Nitrogen is necessary for the synthesis of proteins, nucleic acids and chlorophyll in microalgae [4]. Microalgae assimilated the inorganic nitrogen into biochemically active compounds for their physiological needs. Nitrogen is found to enhance the biomass and lipid production in microalgae and the form of nitrogen

available to the microalgae affects its cellular composition and lipid content. Identification of appropriate nitrogen source and its concentration have influence on microalgal growth as well as lipid accumulation [5-8].

Biodiesel derived from microalgae is one of the promising alternatives of renewable energy due to year round production, higher productivity than terrestrial energy crops. Due to the high growth rate and high oil contents, *Chlorella* spp. showed great potentials for biodiesel production. Heterotrophic and mixotrophic cultures of microalgae have an edge over photo autotrophy as the cell density of phototrophic culture is low which make it hard to be applied in large scale biomass production [9]. Effects of various nutrient sources on biomass and lipid production of *Chlorella* have been reported previously and have been reported to adapt to heterotrophic cultivation [10]. Nitrogen deficiency induced lipid production in microalgae is well documented; however, nitrogen reduction results in decreased carbon dioxide fixation, oxygen evolution, chlorophyll content and biomass production. These results suggest that effect of nitrogen source and concentration is important to attain a better understanding of the behaviour of algal cells for higher biomass and lipid production. Unfolding which nitrogen source influence algal growth and metabolic functions is critical for successful scale up of microalgal culture for biofuel production. This study focused on the identification of most appropriate nitrogen source for the cultivation of *Chlorella vulgaris* in order to improve biomass and lipid productivity.

Materials and Methods

Algal strain and Nitrogen sources

Chlorella vulgaris isolated from sewerage treatment plant, in Bengaluru (13°04'N, 77°58'E) was used to study the effects of nitrogen sources on its growth and lipid productivity. Five different nitrogen sources including ammonium acetate

(NH₄-N), calcium nitrate (NO₃-N), glycine, sodium nitrite (NO₂-N) and urea were used in this study.

Experimental conditions

The experimental set up were carried out in Erlenmeyer flasks under controlled laboratory conditions (temperature 25°C, light intensity of 60 μ mol photons m⁻² s⁻¹ and a light/dark cycle of 12 h/12 h) using Bold's Basal medium. Sodium nitrate was used as the nitrogen source for control and the experimental cultures received nitrogen content equivalent to control cultures. All experiments were carried out in triplicates for a period of 14 days.

Specific growth rate and Biomass productivity

Specific growth rate (μ) of the microalgae was calculated according to the following formula [11].

$$\mu = \frac{\ln(N_t / N_0)}{T_t - T_0}$$

Where, N_t and N₀ are the total cells at the end of log phase (T_t) and start of log phase (T₀), respectively.

Biomass (g L⁻¹) of *C. vulgaris* grown under different nitrogen sources was determined by measuring the optical density of samples at 600 nm (OD₆₀₀) using UV-Vis spectrophotometer. Biomass concentration was then calculated by multiplying OD₆₀₀ values with 0.6, a predetermined conversion factor obtained by plotting OD₆₀₀ versus dry cell weight (DCW). DCW was determined gravimetrically by centrifuging the algal cells (3,000×g, 10 min) and drying.

$$\text{Biomass concentration} = \text{OD}_{600} \times 0.6 \quad \dots\dots\dots \text{Eq. (1)}$$

The biomass productivity (g L⁻¹ d⁻¹) was calculated according to Eq. (2)

$$\text{Biomass productivity} = (B_t - B_0) / (T_t - T_0) \dots\dots\dots \text{Eq. (2)}$$

Yield was calculated from the Eq. (3)

$$\text{Yield (g/L)} = (B_t - B_0) \times \text{Volume of culture} \dots\dots\dots \text{Eq. (3)}$$

Where B_t was the biomass concentration at the end of cultivation period (T_t) and B₀ is the initial biomass concentration at the beginning of the cultivation period (T₀).

Chlorophyll estimation

Chlorophyll contents of the microalga were estimated according to Becker [12]. Algal cells were centrifuged and extracted with acetone overnight. The extract was centrifuged at 3000 × g for 5 min and the chlorophyll content in the supernatant were determined by measuring the optical densities at 645 and 663 nm in a spectrophotometer and then calculated using the Eq. (4).

$$\text{Chl (mg/L)} = 8.02 \times \text{OD}_{663} + 20.21 \times \text{OD}_{645} \dots\dots\dots \text{Eq. (4)}$$

Carotenoids Estimation

Carotenoids were determined by following the procedure of Whyte [13]. Algal cells were centrifuged and treated with KOH (60% w/w). The mixture was homogenized and warmed to 40°C for 40 min and extracted using ethyl ether. The solvent was evaporated followed by resuspending in acetone and the optical density was measured at 444 nm. Total carotenoids were calculated using the Eq.(5).

$$\text{Ct (mg/L)} = 4.32 \times \text{OD}_{444} - 0.0439 \dots\dots\dots \text{Eq. (5)}$$

Lipid productivity

Total lipid of the microalgae was extracted in a Soxhlet extractor using ethyl ether (1:100 w/v) and refluxed at 65°C for 12 h. The extract was distilled at 50°C and the residue was dried at 80°C for 2 h. Lipid content was calculated by dividing the residue weight with the dry cell weight. The lipid productivity was calculated by the Eq. (6).

$$\text{Lipid Productivity (g / L / day)} = \frac{C_{\text{Lipid}} (\text{g / g}) \times \text{DCW (g / L)}}{\text{Time}} \quad (6)$$

Where C_{Lipid} is lipid content of cells, DCW is dry cell weight, and Time is the cultivation period in days

Results and Discussion

Nitrogen source is important in biomass productivity of microalgae and to investigate an optimal nitrogen source for cell growth and lipid accumulation, various nitrogen sources were examined. In general, microalgae utilize ammonium, nitrate, urea and nitrite as the primary nitrogen sources [14,15]. The microalgae *C. vulgaris* was able to respond to nitrogen nutrient changes by inducing specific amino acid transport systems [16].

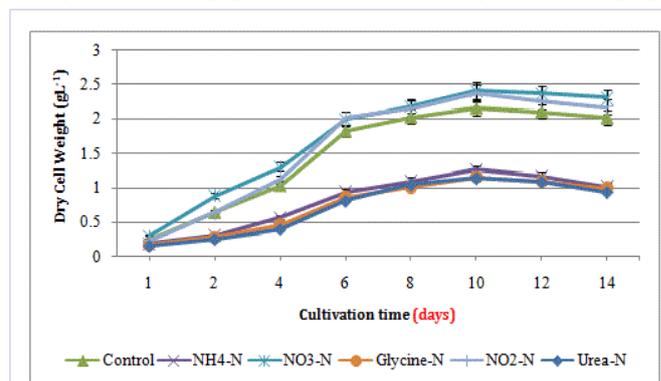


Figure 1: Cell growth of *C. vulgaris* cultivated in varying nitrogen sources

As shown in Figure 1, urea-N, NH₄-N and glycine-N can only support rather poor growth of *Chlorella* under the investigated conditions. NO₃-N in the form of calcium nitrate was the best among the nitrogen sources tested (p < 0.001). NO₃-N led to the highest biomass concentration of 2.41 g L⁻¹ which was more than doubling that obtained with urea and glycine (1.14 and 1.15 g L⁻¹,

respectively). The second other highest biomass concentration was obtained with $\text{NO}_2\text{-N}$ (sodium nitrite) which is 9.5% lesser than $\text{NO}_3\text{-N}$; however, there was no much difference in specific growth rates (μ) (0.082 and 0.081 day^{-1}) under both the nitrogen sources (data not shown). Biomass productivity was calculated using biomass concentration and a highest productivity of $0.34 \text{ g L}^{-1} \text{ day}^{-1}$ was obtained in medium supplemented with $\text{NO}_3\text{-N}$. Both $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ have recorded similar values of $0.29 \text{ g L}^{-1} \text{ day}^{-1}$ (Figure 2).

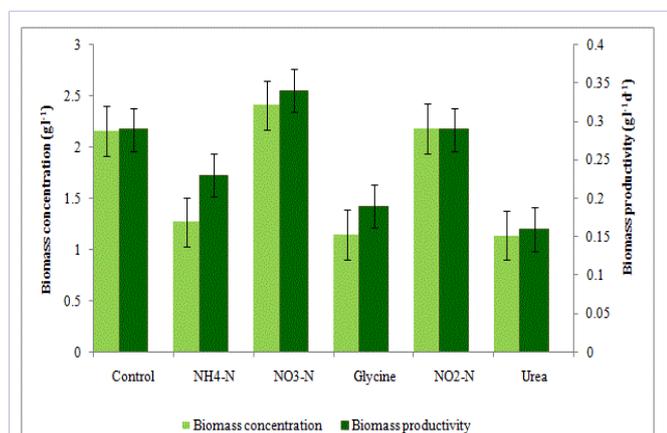


Figure 2: Biomass content and productivity of *C. vulgaris* cultivated in varying nitrogen sources

Urea is preferred as N source for large scale cultivation of microalgae as it is less expensive; however, in this study urea is proved as least effective N source, producing a biomass of $0.16 \text{ g L}^{-1} \text{ day}^{-1}$. Ammonium as N source has barely supported the growth of *C. vulgaris* which might be due to assimilation of ammonium ions that resulted in lower pH. Although microalgae can grow over wide pH, the growth is species dependent. The findings were in correlation with previous studies where ammonium resulted in lesser growth [17,18].

The critical day of biomass growth was identified once the growth rate started to decrease rapidly. Biomass yield was calculated using biomass concentration at the beginning and end of cultivation period (Figure 3). Maximum biomass yield of 16.3 g L^{-1} was obtained with $\text{NO}_3\text{-N}$ followed by $\text{NO}_2\text{-N}$ (13.6 g L^{-1}). Urea and glycine has recorded the least biomass yield at the end of 14 days cultivation period.

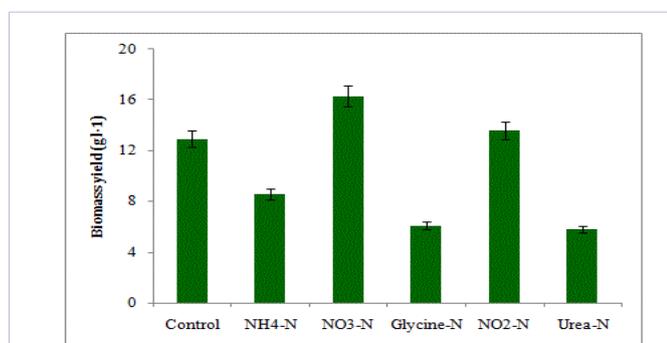


Figure 3: Biomass yield (g L^{-1}) of *C. vulgaris* cultivated in varying nitrogen sources

Chlorophyll is considered as an algal biomass measurement and higher chlorophyll content under $\text{NO}_3\text{-N}$ revealed it as the better source for increasing photosynthetic efficiency which would also promote the biomass production. Carotenoids content of the cells grown under various nitrogen sources were determined spectrophotometrically in which $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ has produced more pigment levels. It was noted that $\text{NO}_3\text{-N}$ in the form of sodium nitrate has produced more carotenoid in algal cells which is new under the investigated conditions. In general, growth parameters in terms of specific growth rate, biomass level and chlorophyll contents were higher in cells grown under calcium nitrate and sodium nitrite throughout the experiment.

The chlorophyll contents of cells grown under different nitrogen sources are shown in Figure 4. From the results, highest chlorophyll content was observed in $\text{NO}_3\text{-N}$ followed by $\text{NO}_2\text{-N}$. In other studies, urea was found to increase the chlorophyll content of microalgae whereas the present study obtained lowest chlorophyll content in urea and glycine [19-20].

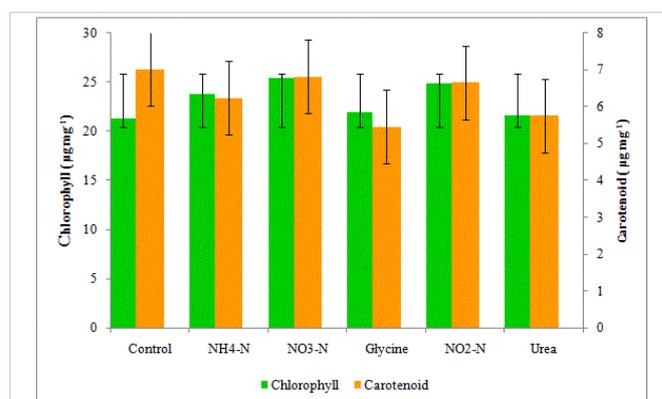


Figure 4: Chlorophyll and carotenoid contents of *C. vulgaris* cultivated in varying nitrogen sources

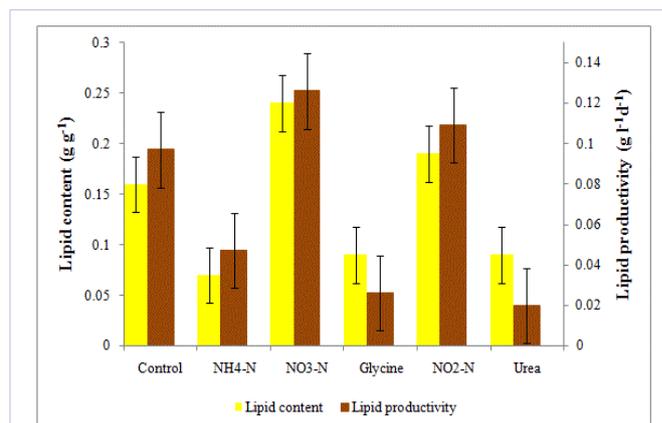


Figure 5: Lipid content and lipid productivity of *C. vulgaris* cultivated in varying nitrogen sources

Figure 5 shows the lipid cell contents (g/g) in dry cells and lipid productivity obtained at different nitrogen sources. In the present study, the highest lipid content (0.24 g/g) was found in $\text{NO}_3\text{-N}$ treatment which was 1.26 fold higher than $\text{NO}_2\text{-N}$ treatment

($p < 0.001$), 3.4 fold higher than $\text{NH}_4\text{-N}$ treatment ($p < 0.001$), 2.6 fold higher than urea-N ($p < 0.01$) respectively (one-way ANOVA, LSD multiple comparisons test). The lipid productivity of cultures provided with $\text{NH}_4\text{-N}$, glycine-N and urea-N remained around 0.020-0.047 ($\text{g L}^{-1} \text{day}^{-1}$). A significant increase to 0.126 $\text{g L}^{-1} \text{day}^{-1}$ in $\text{NO}_3\text{-N}$ and 0.109 $\text{g L}^{-1} \text{day}^{-1}$ in $\text{NO}_2\text{-N}$ supplemented cultures were recorded ($p < 0.01$). The lipid productivity obtained with $\text{NO}_3\text{-N}$ was highest under the investigated condition which was approximately 6.3 times of that of urea-N and 4.8 times of that obtained with glycine-N.

This study finds best nitrogen source for both biomass content and lipid productivity by *C. vulgaris*. In general, there is a contradiction with biomass and lipid productivity and is depending on the initial nutrient concentrations [8,21]. It is well documented that microalgae accumulate more lipid under nitrogen deprived conditions [22-25]. However, the response towards nutrient conditions is highly dependent on the species and strain investigated. Studies on the effect of nitrogen sources on the growth and lipid content in algae are reported and the algal lipid production is greatly affected by nitrogen sources and concentrations (Table 1).

Table 1: Biomass and Lipid productivity of *Chlorella* spp. under various nitrogen sources

Species	Biomass ($\text{g L}^{-1} \text{d}^{-1}$)	Lipid ($\text{mg L}^{-1} \text{day}^{-1}$)	Lipid %	References
<i>Chlorella emersonii</i>	(1.11)/14	50	-	Illman et al. [37]
<i>C. vulgaris</i>	(0.52)/14	14.9	-	Illman et al. [37]
<i>C. protothecoides</i>	19.6	NA	-	Shi et al. [26]
<i>C. protothecoides</i>	(16.8)/8	1214	-	Xiong et al. [38]
<i>C. vulgaris</i>	-	20.44	-	Converti et al. [39]
<i>C. protothecoides</i>	11.7/9	654	50.5	Shen et al. [40]
<i>C. saccharophila</i>	1.1/7	-	37	Isleten-Hosoglu et al. [10]
<i>C. zofingiensis</i>	(0.196)/28	68.1	33.5	Feng et al. [41]
<i>Chlorella</i> sp.	(1.2)/36	600	-	Amin et al. [42]
<i>C. sorokiniana</i>	12.28	2900	31.5	Li et al. [17]
<i>C. sorokiniana</i>	0.218/16	-	61.52	Ramanna et al. [18]
<i>C. ellipsoidea</i>	NA	9.27	-	Gonzalez-Garcinuno et al. [28]
<i>Chlorella</i> sp.	1.673	665	-	Leesing et al. [30]
<i>C. protothecoides</i>	0.605/7	287	48.7	Fei et al. [43]
<i>Chlorella</i> sp.	(0.357)/45	126.25	-	Zhan et al. [29]
<i>C. sorokiniana</i>	NA	883	-	Choi et al. [44]
<i>C. vulgaris</i>	(0.34)/14	126	-	Present study

To obtain biofuel from microalgae in an economically viable way, it is necessary to enhance the microalgal biomass and lipid productivity. Nutrients play key role in affecting both biomass and production and lipid accumulation in microalgae. Various nitrogen sources such as ammonia, nitrate, nitrite, and urea, can be used to cultivate microalgae. Urea [18,26], ammonium [27,28], nitrite [29] and nitrate [30] were found to produce highest biomass and lipid production in various *Chlorella* species. Whereas, nitrate was found as the best nitrogen source for both growth and lipid production in other microalgae [31-36]. In general, decreased N source concentration reduces the biomass productivity and increases lipid content which is due to reduced metabolism rate resulted in channeling of excess carbon towards storage molecules production such as triacylglycerides. Although studies have suggested that nitrogen starvation is most promising culture strategy to increase the lipid productivity, efforts should be taken to improve both biomass and lipid productivity simultaneously.

Conclusion

In this study, $\text{NO}_3\text{-N}$ was demonstrated to be the best nitrogen source for both biomass and lipid-producing potential of *Chlorella vulgaris* under the investigated conditions. Meanwhile, $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ also contributed to algal lipid-producing potential, but not urea-N and glycine-N. Identification of appropriate N source provides an economically feasible strategy to obtain biomass and lipid productivity from microalgae at the same time. However, nutrient concentration plays a vital role in using microalgae as feedstock suggest that further research should be focused on optimizing the concentration of suitable nitrogen source for improving biomass and lipid productivity of *C. vulgaris*.

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