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Research Article

Impact of Different Nitrogen Sources on Growth and Bioactive Compounds in *Chlorella vulgaris* Beijerinck

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Abstract

The present investigation was undertaken to assess the influence of varying nitrogen sources- sodium nitrate ($NaNO_3$) di-ammonium phosphate (DAP), and urea-[$CO(NH_2)_2$] on the accumulation of key biomolecules in *Chlorella vulgaris*. Cultures were maintained under controlled conditions and analyzed on the 7th day of inoculation to quantify chlorophyll-a, b, carotenoid and lipid contents. Among the treatments, cultures supplemented with 100% $NaNO_3$ consistently exhibited the highest concentrations of biomolecules studied. Chlorophyll-a and carotenoid levels peaked at 0.0092 µg/ml and 0.0059µg/ml respectively. Maximum lipid accumulation under 100% $NaNO_3$ was recorded at 0.0074 µg/ml. Cultures treated with DAP exhibited the lowest values for all parameters. These findings indicate that $NaNO_3$, particularly at full strength, is the most efficacious nitrogen source for enhancing pigment synthesis and lipid accumulation in *Chlorella vulgaris*, thereby highlighting its potential for optimizing algal biomass production in biotechnological applications.

Keywords: Algae; Biomolecules; Biofuels; DAP; $NaNO_3$; Urea

Abbreviations

DAP: Di Ammonium Phosphate; ROS: Reactive Oxygen Species.

Introduction

Microalgae are a diverse group of photosynthetic microorganisms that have gained considerable attention due to their rapid growth, high photosynthetic efficiency, and ability to synthesize a wide range of valuable biomolecules, including pigments, proteins, carbohydrates, lipids, vitamins, and bioactive compounds [1]. These organisms play a vital role in global carbon cycling by fixing atmospheric carbon dioxide and converting it into organic biomass through photosynthesis [2]. In recent years, microalgae have emerged as promising bio-resource for applications in biofuel production, nutraceuticals, pharmaceuticals, wastewater treatment, carbon sequestration, aquaculture, and sustainable agriculture [3]. Their ability to grow under diverse environmental

conditions while utilizing inorganic nutrients makes them ideal candidate for environmentally friendly biotechnological processes.

Among the various environmental and nutritional factors regulating algal growth, nitrogen is considered one of the most essential factor [4]. Nitrogen is a structural component of amino acids, proteins, nucleic acids, chlorophyll molecules, enzymes, ATP, and numerous secondary metabolites [5]. It directly influences cellular metabolism, photosynthetic efficiency, biomass accumulation, and the synthesis of commercially important metabolites [6]. Since nitrogen availability governs both cell division and metabolic activity, its concentration and chemical form significantly affect the physiological and biochemical characteristics of algal cells [7]. Microalgae can assimilate nitrogen in several inorganic and organic forms, including nitrate (NO_3^-), ammonium (NH_4^+), urea [$CO(NH_2)_2$], nitrite (NO_2^-), and certain amino acids [8]. However, different nitrogen sources vary considerably in their uptake mechanisms, assimilation pathways, metabolic costs, and influence on cellular metabolism [9]. Consequently, the selection of an appropriate nitrogen source plays a crucial role in optimizing algal cultivation for maximum biomass productivity and enhanced accumulation of valuable metabolites such as pigments and lipids [10].

The commonly used nitrogen fertilizers, sodium nitrate, DAP, and urea are widely employed in laboratory culture media and agricultural practices. These nitrogen sources differ in chemical composition, nitrogen availability, assimilation efficiency, and associated nutrient supply. Sodium nitrate primarily provides nitrate nitrogen, DAP supplies both ammonium nitrogen and phosphorus, whereas urea serves as an organic nitrogen source that is rapidly hydrolyzed into ammonium and carbon dioxide by the enzyme urease [11]. These differences substantially influence nutrient uptake, photosynthetic activity, cellular metabolism, and biochemical composition of algal cells [12]. The biochemical responses of algae to sodium nitrate, DAP, and urea are often species-specific because different algae possess varying nitrogen transport systems, enzymatic activities, metabolic capacities, and regulatory mechanisms [13]. Freshwater green algae exhibit considerable variation in nitrate reductase activity, urease expression, ammonium tolerance, and phosphorus utilization [14]. Therefore, comparative evaluation of multiple nitrogen sources is essential for developing species-specific cultivation strategies aimed at improving biomass production and metabolite accumulation.

Optimization of nitrogen based nutrition has significant implications beyond laboratory cultivation. In large-scale commercial algal production systems, nitrogen fertilizers constitute a substantial proportion of operational costs [15]. Selecting an economical nitrogen source capable of maximizing growth while enhancing pigment and lipid synthesis can significantly improve the economic feasibility of algal biotechnology [16]. Furthermore, understanding the physiological responses of algae to agricultural fertilizers such as DAP and urea may facilitate the development of integrated cultivation systems utilizing agricultural runoff, wastewater, or nutrient-rich effluents, thereby contributing to sustainable resource utilization and environmental remediation [17].

The present study was therefore undertaken to investigate the comparative effects of three commonly used nitrogen sources-sodium nitrate ($NaNO_3$), di-ammonium phosphate (DAP), and urea [$CO(NH_2)_2$] on the growth and biochemical composition of algae. Particular emphasis was placed on evaluating changes in biomass production, chlorophyll content, carotenoid content, and lipid accumulation under different nitrogen treatments. The study also aims to identify the most suitable nitrogen source for promoting optimal algal growth while simultaneously enhancing the synthesis of photosynthetic pigments and lipids. The findings are expected to contribute to a better understanding of nitrogen metabolism in algae and provide valuable information for optimizing culture media in applications related to biofuel production, nutraceutical development, wastewater treatment, and sustainable algal biotechnology.

Material and Methods

Culture collection and maintenance

The study was carried out using *Chlorella vulgaris* Beijerinck sourced from the Germplasm Collection Center of the *Chlorella vulgaris* Biotech Laboratory, Department of Botany, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India. The microalgae were cultured under controlled laboratory conditions in nutrient media BG-11 [18] containing different nitrogen sources to assess their influence on algal growth and synthesis of bioactive

Table 1: Showing concentrations of different nitrogen sources

S. No	Treatment	Conc. 100%	Conc. 75%	Conc. 50%	Conc. 25%
1.	NaNO3	1.5 g/l	1.125 g/l	0.750 g/l	0.375 g/l
2.	Urea	0.53 g/l	0.398 g/l	0.265 g/l	0.133 g/l
3.	DAP	1.17 g/l	0.874 g/l	0.583 g/l	0.291 g/l

metabolites.

Media preparation

The initial culture was maintained for seven days with a light-dark cycle of 14:10 hours, and then inoculated into media with varied concentrations of nitrogen sources *viz.* sodium nitrate ($NaNO_3$), di-ammonium phosphate (DAP), and urea [$CO(NH_2)_2$] at four concentration levels (100%, 75%, 50%, and 25%), in triplicates (Table-1).

Cultivation

The culturing was performed in 100 ml of nutrient medium in 150 ml conical flasks, and the cells were harvested on 7th day from inoculation.

Chlorophyll estimation

Chlorophyll content was measured using the Lichtenthaler and Wellburn method, involving homogenization of algal biomass in 90% acetone, followed by centrifugation at 5000 rpm for 10 minutes. The optical density (OD) was recorded at 663 and 645 nm [19].

Carotenoids estimation

Carotenoids were measured using the Jensen method, involving homogenization of algal biomass in 90% acetone, followed by centrifugation at 5000 rpm for 10 minutes. The optical density was estimated at 453 nm [20].

Lipid estimation

Lipids were extracted using the Bligh and Dyer method. The technique employs liquid -liquid extraction utilizing a ternary solvent mixture of chloroform, methanol, and water. Lipids dissolve in chloroform, whilst non-lipid components are detained in the aqueous methanol phase [21].

Statistical analysis of data

Statistical analysis of data was done in Microsoft Excel 2007 [22].

Result

The results demonstrated chlorophyll-a accumulation under different nitrogen sources (Figure 1). $NaNO_3$ showed a concentration-dependent decline at 100% from 0.0092 µg/ml to 0.0072 µg/ml at 25%, but remained the most effective nitrogen source throughout the experimental range. In contrast, urea exhibited an increasing trend from 0.0058 to 0.0069 µg/ml, while DAP increased more markedly from 0.0022 to 0.0054 µg/ml as the concentration of urea and DAP decreased from 100% to 25%. These findings suggest that nitrate-based nitrogen was more favorable for chlorophyll-a synthesis at higher nutrient availability, whereas

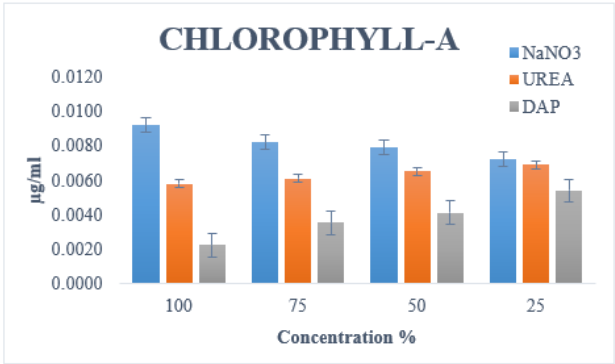

Figure 1: Chlorophyll- a content in $NaN0_3$, Urea, DAP treatment.

reduced concentrations of urea and DAP promoted comparatively greater pigment accumulation.

The high chlorophyll-b content observed with 100% $NaNO_3$ suggests that nitrate was particularly effective in supporting chlorophyll-b synthesis under full nitrogen availability. However, the reduction in chlorophyll-b at lower $NaNO_3$ concentrations indicates that chlorophyll-b synthesis may have been constrained by reduced nitrate availability (Figure 2). Conversely, the increasing chlorophyll-b content under urea and DAP with decreasing concentration suggests that lower concentrations of these nitrogen sources were more favorable for pigment accumulation. Overall, 100% $NaNO_3$ concentration resulted in the maximum chlorophyll-b content, while at lower concentrations the differences among the three nitrogen sources became relatively small. At 25% urea and $NaNO_3$ concentration, showed nearly comparable chlorophyll-b levels, indicating that reduced concentrations of urea may effectively support chlorophyll-b synthesis.

Total chlorophyll content showed clear differences among the $NaNO_3$, Urea, and DAP treatments across the tested concentrations. $NaNO_3$ consistently recorded the highest chlorophyll content, followed by Urea, while DAP generally showed the lowest values

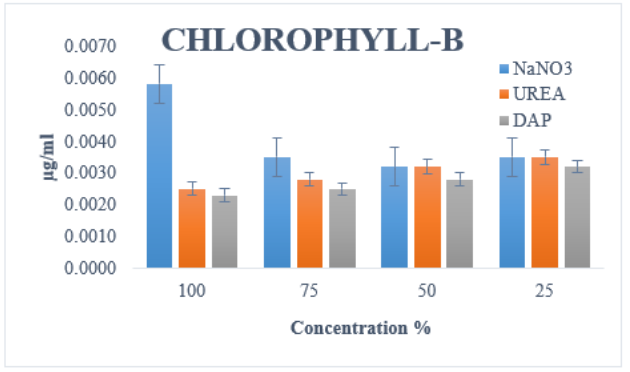

Figure 2: Chlorophyll- b content in $NaN0_3$, Urea, DAP treatment.

(Figure 3). At 100% concentration, $NaNO_3$ produced approximately 0.015 µg/ml, compared with 0.0082 µg/ml for Urea and 0.0045 µg/ml for DAP. At 75% concentration, chlorophyll content was approximately 0.012, 0.0088, and 0.0060 µg/ml for $NaNO_3$, Urea, and DAP, respectively. At 50%, the values were approximately 0.011, 0.0097, and 0.0068 µg/ml, respectively. At 25%, $NaNO_3$ and Urea showed relatively similar values (~0.0105 and ~0.0100 µg/ml), whereas DAP remained lower (~0.0085 µg/ml). $NaNO_3$ was the most effective treatment for chlorophyll production, particularly at higher concentrations. Urea showed a moderate response, while DAP produced comparatively lower chlorophyll content. However, DAP showed an increasing trend as its concentration decreased, suggesting that its response pattern differed from those of $NaNO_3$ and Urea.

The carotenoid content showed considerable variation among the $NaNO_3$, Urea, and DAP treatments at different concentrations. Overall, $NaNO_3$ recorded the highest carotenoid content at all concentrations, while DAP generally showed the lowest values (Figure 4). At 100% concentration, $NaNO_3$ produced the highest carotenoid content (0.0038 µg/ml), followed by Urea (0.0014 µg/ml) and DAP (0.0012 µg/ml). At 75% concentration, $NaNO_3$ remained highest (0.0035 µg/ml), whereas Urea increased to 0.0019 µg/ml and DAP showed 0.0013 µg/ml. At 50% concentration, $NaNO_3$ recorded 0.0034 µg/ml, followed by Urea (0.0022 µg/ml) and DAP (0.0014 µg/ml). At 25% concentration, $NaNO_3$ decreased further to 0.0031 µg/ml, while Urea showed a marked increase to 0.0029 µg/ml, approaching the $NaNO_3$ value. DAP also increased to 0.0015 µg/ml, but remained the lowest among the three treatments. The results indicate that $NaNO_3$ was the most effective treatment for carotenoid accumulation, maintaining the highest values from 100% to 25% concentration. Urea showed a progressive increase in carotenoid content as the concentration decreased, with its value at 25% becoming almost comparable to $NaNO_3$. DAP consistently produced the lowest carotenoid content,

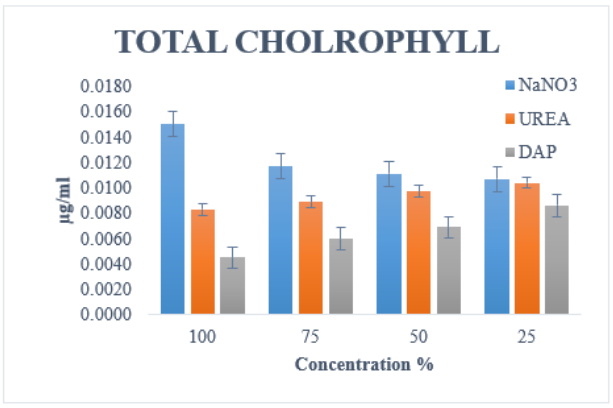

Figure 3: Total chlorophyll content in $NaN0_3$, Urea, DAP treatment.

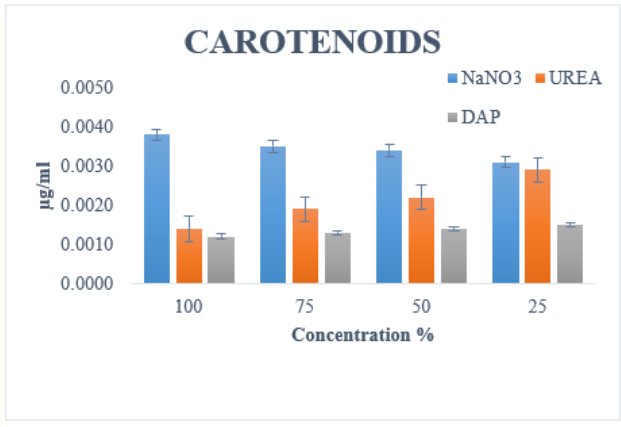

Figure 4: Carotenoid content in $NaN0_3$, Urea, DAP treatment.

although a slight increasing trend was observed with decreasing concentration. Thus, the overall order of carotenoid content was $NaNO_3$ > Urea > DAP, with the difference between $NaNO_3$ and Urea becoming smaller at lower concentrations.

The lipid content showed considerable variation among the $NaNO_3$, Urea, and DAP treatments under different concentrations (Figure 5). Overall, $NaNO_3$ recorded the highest lipid content at 100%, 75%, and 25% concentrations, whereas Urea showed the highest value at 50% concentration. DAP consistently recorded the lowest lipid content across all concentrations. At 100% concentration, $NaNO_3$ showed the highest lipid content (0.0054 mg/ml), followed by Urea (0.0028 mg/ml) and DAP (0.0015 mg/ml). At 75% concentration, $NaNO_3$ again recorded the highest value (0.0049 mg/ml), while Urea showed 0.0029 mg/ml and DAP showed 0.0013 mg/ml. At 50% concentration, a different trend was observed. Urea recorded the highest lipid content (0.0032 mg/ml), followed by $NaNO_3$ (0.0015 mg/ml) and DAP (0.0012 mg/ml). At 25% concentration, $NaNO_3$ increased to 0.0041 mg/ml, followed by Urea (0.0035 mg/ml) and DAP (0.0011 mg/ml). The results indicate that $NaNO_3$ generally promoted the highest lipid accumulation, particularly at 100% and 75% concentrations. However, at 50% concentration, Urea produced the highest lipid content, exceeding $NaNO_3$ by a considerable margin. At 25%, $NaNO_3$ again showed the highest value, with Urea showing a relatively close response. DAP consistently exhibited the lowest

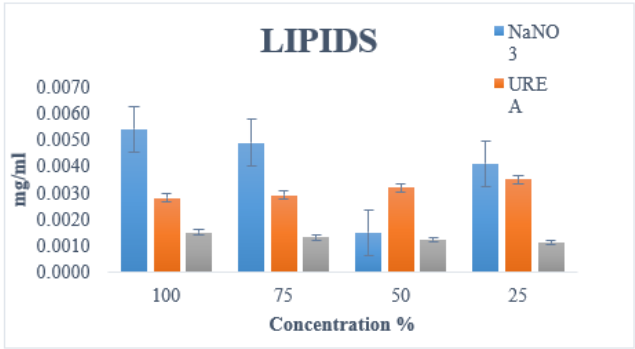

Figure 5: Lipid content in $NaNO_3$, Urea, DAP treatment.

lipid content at all concentrations. The comparative results obtained in lipid assimilation was $NaNO_3$ > Urea > DAP, except at 50% concentration, where the order changed to Urea > $NaNO_3$ > DAP. These results suggest that the effect of fertilizer type on lipid accumulation was concentration-dependent.

Discussion

The markedly elevated levels of chlorophyll-a and chlorophyll-b under 100% $NaNO_3$ suggest that nitrate serves as a sufficient and easily assimilable nitrogen source for chlorophyll production and chloroplast growth. Nitrogen is a fundamental structural element chlorophyll molecules and photosynthetic proteins; hence, enough available nitrate promotes pigment production and optimization photosynthetic efficiency [23,24].

Carotenoids accumulation dramatically enhanced in $NaNO_3$ treated cells compared to those treated with DAP and urea. Carotenoids solve as accessory pigment in light harvesting and are essential for safeguarding photosynthetic system from oxidative stress by neutralizing reactive oxygen species [25]. The evaluated carotenoid level noted under nitrate nutrition indicates improved psychological condition, increased photosynthetic efficiency and heightened metabolic activity in *Chlorella* sp. Comparable findings has been shown in microalgae grown under optimal nitrogen circumstances, where sufficient nutrient enhanced the pigment production and overall cellular vitality [26].

Lipids formation was maximized in cultures enriched with 100% $NaNO_3$, nitrogen constraint is generally associated with the buildup of neutral lipids in micro algae; nevertheless, extreme nitrogen deprivation typically hinders cellular growth and demises total lipid output due to reduced biomass yields [27]. The current study demonstrated that adequate nitrate availability facilitated swift biomass formation and concurrently increased lipid biosynthesis, leading to an elevated total lipid content. These findings suggest that optimal nitrogen nutrition enhances lipid productivity by sustaining cellular growth and metabolic functions, aligning with earlier studies that highlights the necessity of optimizing nutrient availability instead of applying extreme nutrients [27,28].

Conversely, DAP treatments, especially at 25% dose, yielded markedly reduced levels of pigments, fats and carbohydrate content. This decline may be ascribed to diminished nitrogen availability or decreased nitrogen assimilation efficiency, which can handle chlorophyll synthesis, reduce photosynthetic capacity, and ultimately restrict the generation of cellular metabolites. While urea

facilitated superior biochemical accumulation compared to DAP, its efficacy was still lower to that of sodium nitrate. The disparities in nitrogen assimilation pathways, enzymatic requirements, and the possible accumulation of ammonium during urea hydrolysis may lead to the somewhat diminished biochemical production noted in urea treated cultures [29].

Conclusion

The present study was conducted to evaluate the influence of different nitrogen sources- sodium nitrate ($NaNO_3$), di-ammonium phosphate (DAP), and urea [$CO(NH_2)_2$] on the production of key biomolecules such as chlorophyll-a, chlorophyll-b, carotenoids and lipids in *C. vulgaris* across different concentrations i.e. 100%, 75%, 50% and 25%. The findings demonstrated that the type of nitrogen source significantly affects the metabolic activities and biosynthetic pathway of *C. vulgaris*. which in turn influence the concentration of valuable biomolecules. Among the tested in nitrogen sources, $NaNO_3$ consistently produced superior results in term of pigment and lipid content, highlighting its suitability for enhanced biomass and biochemical production in *C. vulgaris* cultivation systems.

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