Optimization of macronutrient kinetics for biomass production in *Nostoc calcicola*

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Abstract: Addition of nutrients is a promising strategy for maximizing growth of *Nostoc calcicola*. To assess the feasibility of Allen and Arnon’s (AA) media addition to increase the biomass productivity, (0, 2.5, 5, 7.5 ml of 10x media concentrate - MC) was added to aerated culture every six days, in two separate conditions i.e., single harvest (SH) and continuous harvest (CH) after 15th day. Results show that with addition of 5 ml of MC produced maximum amount of biomass is 1.32 g/L and 2.88 g/L for SH and CH respectively. These results show that with addition of 5 ml of MC to an aerated culture every six days with continuous biomass harvesting leads to maximum growth of *Nostoc calcicola* @25°C

Keywords: *Nostoc calcicola*, macronutrients, Biomass production

1. Introduction

The rapid surge of CO₂ content in the environment is viewed as a primary driver of greenhouse effect leading to extreme temperatures around the globe. Photosynthetic microorganism having the capability of fixing CO₂ in atmosphere provides a chance of economically growing such organisms in industrial scale [1].

Algae belong to a large group of simple photosynthetic organisms. They are subdivided into two major categories based on their size. Microalgae, are small free-living microorganisms that can be found in a variety of aquatic habitats. They are able to thrive in freshwater, brackish, marine and hypersaline aquatic environments and have been reported in desert crust communities thereby being able to endure temperature extremes and low water availability [2,3].

The main advantages of using micro algal organisms in a variety of industrial applications are: they grow rapidly and have a higher solar conversion efficiency than most terrestrial plants. It can be harvested batch-wise or continuously almost all year round. Algal production facilities can be collocated on otherwise non-productive, non-arable land. It can utilize salt and waste water sources that cannot be used by conventional agriculture [4]. It can use waste CO₂ sources thereby potentially mitigating the release of greenhouse gases into the atmosphere [5]. It can produce a variety of feedstocks that can be used to generate nontoxic, biodegradable biofuels and valuable co-products[6]. Microalgae have current applications in the production of human nutritional supplements and specialty animal feeds. Microalgae are currently cultivated as a source of highly valuable molecules such as polyunsaturated fatty acids (PUFAs) and pigments such as β-carotene and astaxanthin. Currently, commercial production of
microalgae biomass is limited to a few species, such as *Spirulina, Chlorella,* and *Dunaliella,* cultivated in open, CO₂ fertilized ponds for high value nutritional products [6,7].

*Nostoc* environments are diverse and widespread over the globe; isolates have been found in fresh water, soils, and both extremely cold and extremely arid habitats. Their role as a nitrogen fixer in terrestrial ecosystems allow them to maintain symbiotic interactions with organisms including fungi, lichen, mosses, and ferns. Some types of *Nostoc* are edible, and are even considered delicacies in some Chinese regions. It was reported that a strain of *Nostoc* (*N. flagelliforme*) and its exopolysaccharides (EPS) in culture media contain antitumor and antiviral components such as acidic polysaccharide *nostoflan* [8-11]. *Nostoc calcicola* is also used for heavy metal bio sorption.

The major drawback for growing microalgae is that it requires lots of water for biomass production. This could be compensated by adding nutrients to the culture continuously. It is been reported that *Nostoc* can grow under elevated CO₂ conditions and has shown increase in biomass productivity with addition of phosphate [12-15]. However no study has been carried out for addition of entire media nutrients as continuous culture.

Aim of this study is to increase the biomass productivity with varying media concentration by adding Allen’s and Arnon’s (AA) media (0, 2.5, 5, 7.5 ml of MC) as continuous culture periodically. The effects of media with single harvest (SH) and continuous harvest (CH), on the biomass productivity and uptake of major nutrients like magnesium and phosphorous were investigated in this study. The study would provide important information for commercial use of *N. calcicola* for biomass production.

2. Experimental Methods

2.1 Procedure

The filamentous cyanobacterium *Nostoc calcicola* (wild Type) was grown in Allen and Arnon’s (AA) medium, was maintained in a culture room illuminated with cool daylight fluorescent tubes (14.4 W/m²) at 25±2 °C. The media was prepared for 32 liters and was transferred to 8 containers of 4 liter capacity. About 140 ml of each strain of *N. calcicola* were inoculated into these four containers. The exponentially grown *N. calcicola* cells were partially harvested every three days by removing 13 ml of solution from each of these flasks and centrifuging at 3,000 rpm at RT for 10 min in 15 ml pre-weighed centrifuging containers (REMI Compufuge, India). The 10 ml of supernatant was filtered and stored in dried, pre-weighed beakers for TDS measurement. The remaining solution was removed and the algae was dried in oven at 80°C, for 24 hours. The dried containers weight along with the algae’s weight is measured to find the biomass produced.

A 10x AA media concentrate was fed to each container with 0, 10, 20, 30 ml every six days respectively for first 4 containers and the same is harvested at the end of 30th day, which is called single harvest (SH). Another set of experiments of the same condition of SH, was carried out for 30 days. Such that half of the biomass was removed after 15 days for every 6 days thereafter for the other 4 containers, which is called continuous harvest (CH).

The pH of the media, conductivity, TDS was found using pH and conductivity meter for every set of reading for both SH and CH experiments. All the experiments carried out at 25°C.

2.2 Culture conditions

The most essential macronutrients for the growth of microalgae are magnesium and phosphorous. Magnesium is building block of chlorophyll, which makes leaves appear green and its deficiency could
lead to lower yield. Phosphorous in other hand is used for transfer of energy, metabolic regulation and protein activation by molecules like (ATP, ADP, AMP), nucleic acids, phosphates and phospholipids.

Dissociated cells (separated filament without capsule) of *N. calcicola* were obtained according to the previous methods [16-18]. Components of the culture medium Allen and Arnon’s (AA media) used in this study are as follows: (CaCl$_2$ 0.055 g/L, NaCl 0.234 g/L, MgSO$_4$$\cdot$7H$_2$O 0.248 g/L, K$_2$HPO$_4$ 0.348 g/L, H$_3$BO$_3$ -0.05 mg/L, ZnSO$_4$$\cdot$4H$_2$O 0.05 mg/L, CuSO$_4$ 0.02 mg/L, Fe EDTA 1 ml/L, CoNO$_3$$\cdot$6H$_2$O 0.01 mg/L, MoO$_3$ -0.1 mg/L). Filtered air was used to grow the cultures.

2.3 ICP Analysis

The concentrations of Magnesium (Mg) and Phosphorous (P) were determined by Inductively Couples Plasma-Optical Emission Spectrometer (ICP-OES), Perkin Elmer Optima 7000 DV. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. ICP-OES was first calibrated with standard solutions in the range covering concentrations of likely to be found in samples. Samples were analyzed in triplicates to maintain reproducibility [19-20].

3. Results and discussion

The amount of Biomass, pH of the culture and TDS of the media during 30 days of cultivation for bottle aeration is given in this section.

3.1 Biomass Harvest

In the SH experiments, the biomass production increased gradually as the amount of the continuous media input increases. The maximum biomass produced at the end of 30$^{th}$ day was found for 5 & 7.5 ml of MC (1.33 g/L) which was 15.6% greater than that of control. (Fig 1) As per Hexin Lv, et al.[14] the dry cell weight of *N. flagelliforme* after 30 days of aeration, complete harvesting gave 1.45 g/L which is 9 % more production than the present work.

![Figure 1](image)

Figure 1: Biomass produced during cultivation at different volumes of media addition for single harvest (SH) experiments. (O control, □ 2.5 ml of MC, Δ 5 ml of MC, ◊ 7.5 ml of MC).

However in the CH experiments, biomass production increases till 15$^{th}$ day, after 50% harvest, biomass decreases till 18$^{th}$ day and increases again. A cyclic pattern appears in which, after every harvest (15, 21, 27$^{th}$ day) the reduction of biomass is less pronounced as that of the previous one. At the end of 30$^{th}$ day, the maximum biomass produced is achieved by continuous addition of 5 & 7.5 ml of MC (0.72 & 0.7 g/L respectively) which is 39.2% greater than that of control (0.51 g/L). (Fig 2)
Figure 2: Biomass produced during cultivation at different volumes of media addition for continuous harvest (CH) experiments. (O control, □ 2.5 ml of MC, △ 5 ml of MC, ◊ 7.5 ml of MC).

The total amount of biomass harvested was much greater for CH compared to SH experiments. It was observed that harvest was maximum for 5 ml of MC (Fig 3). The cumulative biomass produced for continuous harvesting was 2.91 g/L, for 5 ml of MC, which 119% higher than the maximum biomass obtained by the SH experiments. This could be attributed to amount of algal cells present in the media (cell dilution ratio). If the biomass is not continuously harvested, the cell dilution ratio increases and there requisite media is not present for the cell to grow further, due to which it plateaus. However if algae is continuously harvested, the cell dilution ratio is maintained at an optimum which creates further scope for growth.

Figure 3: Effect of AA media addition on cumulative harvested biomass

3.2 pH, TDS and Conductivity of the culture

The medium pH is also the comprehensive reflection of the alkaline compounds secreted by the cells and the amount of media present in the solution. Excessive alkalization of media is due to the alkaline property of K₂HPO₄. For the SH and CH experiments, pH of the media resembles a bell shaped curve. In the growth phase pH increases due to exo poly saccharides (EPS) which was believed to increase with addition of media concentrate, leading to its alkalinity. The pH of media in CH experiments reached a maximum on 18th day in the range of 8.77 to 9.01 pH. However, the pH declined drastically for all cases after 18th day onwards. This could be attributed to the onset of death phase in algae (Fig 4). During the
death phase, EPS produced by the algae decreases and the amount of CO$_2$ sequestered by algae from atmosphere remains the same thereby decreasing its alkalinity [7].

TDS and conductivity were measured using conductivity meter. Both the parameters followed a decreasing trend and the decrease was found to be less pronounced as the amount of media added increased. A similar pattern was observed when biomass was continuously harvested from the system [21].

**Figure 4:** Effect of various parameters such as pH, conductivity, TDS, during cultivation at different media concentrate. (O control, □ 2.5 ml of MC, Δ 5 ml of MC, ◊ 7.5 ml of MC).
3.3 Elemental Analysis

Elemental analysis of solution were done periodically to estimate the concentration of free magnesium and phosphorous in the media. The concentration of phosphorous in all conditions is greater than magnesium as the media contains higher amount of K$_2$HPO$_4$ than MgSO$_4$. Two general trends were found out from the analysis. Firstly, if the amount of media added to the culture increases, the concentration of nutrients also increase and if the free nutrients in the culture deviates, the amount of biomass produced decreases. This shown in graph 4, when the free nutrients [(P1,P2),(Mg1,Mg2)] in the culture for 5 ml of MC is [(58, & 52),(49 & 46)] ppm respectively. The biomass produced is maximum in both cases. However under 2.5 ml of MC addition the free nutrients in culture deviate quite considerably and the biomass produced is quite low as shown in Fig 1 & 3.

However if algae is continuously harvested, the cell dilution ratio is maintained at an optimum which creates further scope for growth. This is proved by testing free phosphorous present in the solution. As the amount of media added continuously, the amount of free P, Mg increases as shown in the Fig 5. Over addition of nutrients leads to toxicity and inhibiting the overall growth of *Nostoc calcicola*.

![Graphs showing elemental analysis](image)

**Figure 5:** ICP analysis of phosphorus (P) and magnesium (Mg) under various conditions (O P-SH, □ P-CH, ◊ Mg-SH, Δ Mg-CH)

This is proved from ICP analysis that if biomass is continuously harvested, the nutrients are consumed more than only harvesting in the end. Also continuously harvesting the algae partially gives scope for growing longer than harvesting in the end. Considering all the parameters for various experiments performed, we concluded that 5 ml of MC is the most favorable growth condition for *Nostoc calcicola*. 
4. Conclusion

With the CH experiments, it is able to achieve more biomass compared to Hexin Lv, et al [18]. In this present study, the growth characteristics of biomass were investigated under continuous addition of media with and without continuous harvest of *Nostoc calcicola* respectively. 5 ml of MC is the optimum amount by evaluating many parameters such as dry cell weight, cumulative biomass harvested, TDS and pH respectively.

5. Acknowledgements

The authors thank Birla Institute of Science and Technology – Pilani, Dubai Campus for funding Chemical & Biotech Dept. for this research and also for allowing access to sophisticated instruments.

6. References:

[1] Gao K, Qiu B, Xia J, Yu A 1998 Light dependency of the photosynthetic recovery of *Nostoc flagelliforme*. *J. appl. Phycol*. **10** 55–58.

[2] Scherer S, Ernst A, Chen TW, Böger P 1984 Rewetting of droughtresistant blue-green algae: time curse of water uptake and reappearance of respiration, photosynthesis, and nitrogen fixation. *Oecologia* **62** 418–423.

[3] Gao KS, Ye CP 2003 Culture of the terrestrial cyanobacterium, *Nostoc flagelliforme* (Cyanophyceae), under aquatic conditions. *J Phycol* **39** 617–623.

[4] Hu M, Zhao Y, Zhang Z, Zhao P 1987 A Preliminary study on natural soil environment for hair algae growth and the artificial culture. Acta Agriculturae Univesitatis Gansu **3** 76–81 (Chinese, with English summary).

[5] Gao K, Yu A 2000 Influence of CO₂, light and watering on growth of *Nostoc flagelliforme* mats. *J Appl Phycol* **12** 185–189.

[6] Gao K 1998 Chinese studies on the edible blue-green alga, *Nostoc flagelliforme*: a review. *J Appl Phycol* **10** 37–49

[7] De Philippis, R., C. Sili, R. Paperi, and M. Vincenzini 2001 Exopolysaccharide producing cyanobacteria and their possible exploitation: A review. *J. Appl. Phycol*. **13** 293-299.

[8] Liu XJ, Chen F 2003 Cell differentiation and colony alteration of an edible terrestrial cyanobacterium *Nostoc flagelliforme*, in liquid suspension cultures. *Folia Microbiol* **48** 619–626

[9] Kanekiyo K, Le JB, Hayashi K, Takenaka H, Hayakawa Y, Endo S, Hayashi T 2005 Isolation of an antiviral polysaccharide, *Nostoflan*, from a terrestrial cyanobacterium, *Nostoc flagelliforme*. *J Nat Prod* **68** 1037–104.

[10] Su J, Jia S, Chen X et al 2008 Morphology, cell growth, and polysaccharide production of *Nostoc flagelliforme* in liquid suspension culture at different agitation rates. *J Appl Phycol* **20** 213–217.

[11] Lv H, Jia S, Xiao Y et al 2013 Effect of NaNO₃ on the growth and photosynthesis of *Nostoc flagelliforme* cells. *China Brewing* **32** 13–16
[12] Su J Y (2006) Study on the Photoautotrophic Cultivation of Nostoc flagelliforme Cells. Ph.D. Thesis. Tianjin University of Science and Technology, Tianjin, China

[13] Jia S R, Su J Y, and Qiao C S 2006 Method of Nostoc flagelliforme cells cultivation and the Polysaccharides production. China Patent ZL 03119101.0.

[14] Lv H, Jia S, Xiao Y et al 2014 Growth characteristics of Nostoc flagelliforme at intermittent elevated CO₂ concentrations. *Phycol Res* 62 250–256.

[15] Lee N K, Oh H M, Kim H S, Ahn C Y 2017 Higher production of C-phycocyanin by nitrogen-free (diazotrophic) cultivation of Nostoc sp. NK and simplified extraction by dark-cold shock. *Bioresour. Technol.* 227 164–170.

[16] Berdalet E, Latasa M, Estrada M 1993 Effects of nitrogen and phosphorus starvation on nucleic acid and protein content of Heterocapsa sp. *J Plankton Res* 16 303–316.

[17] Marschner H 1995 Mineral nutrition of higher plants. Academic press, San Diego

[18] Hexin L, Xia F, Jia S, Cui X and Yuan N 2015 Effects of K₂HPO₄ on the Growth of Nostoc Flagelliforme in Liquid Media with Different Carbon Sources. *Advances in Applied Biotechnology*. 332 407-415

[19] Murphy J and Riley J 1962 A modified single solution method for the determination of phosphate in natural waters. *Anal Chim Acta* 27 31–36

[20] Parikh A and Madamwar D 2006 Partial characterization of extracellular polysaccharides from cyanobacteria. *Bioresour. Technol.* 97 1822-1827.

[21] Gao K, Ye C 2003 Culture of the terrestrial cyanobacterium, Nostoc flagelliforme (cyanophyceae), under aquatic conditions. *J Phycol* 39 617–623