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RESEARCH ARTICLE

Optimization of sodium nitrate concentration for growth and biodiesel potential in *Phormidium* sp.

■ **ANIL KUMAR JAISWAL AND RICHA SHARMA**

SUMMARY

Phormidium sp. is thermophilc, filamentous, non-heterocyst, blue green algae (Cyanobacteria) belongs to Phormidiaceae family. They can grow in variety of habitat as fresh water, sewage water, waste water and rice field. In the present study, the effect of *Phormidium* sp. on growth biomass with TAG's accumulation at different concentrations of sodium nitrate ($NaNO_3$) has been discussed. Biomass productivity under sodium nitrate enriched BG11 media for *Phormidium* exhibited an increment in growth rate and biomass production at lower concentration of sodium nitrate under 21days. Biomass production was almost triple from the original concentration under different treatment. The results indicated that optimum concentration for the highest biomass and growth rate of the *Phormidium* sp. was 1.5 g/lit of $NaNO_3$ while the lipid content was increased (4.3 ml) under low concentration (0.25g/lit) of $NaNO_3$ which indicates that nitrogen deficiency provokes stress condition which increases the accumulation of lipids. Thus, the study concluded that nitrogen is necessary for the biomass production but to enhance the biodiesel production it should be grown in lower concentration of nitrogen enrichment.

Key Words : Biomass, TAG, Sodium nitrate, BG-11

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Microalgae are prokaryotic photosynthetic micro-organisms which can grow rapidly in specific and also in adverse conditions due to their unicellular or simple multicellular structure (Aslan and Kapdan, 2006; Pratoomyot *et al.*, 2005 and Mata *et al.*, 2010). Microalgae can convert carbon dioxide from the air and light energy through photosynthesis to organic matter as they are mostly photoautotrophic in their nourishment (Spolaore *et al*., 2006). Like plants, microalgae use sunlight to produce oils but they do so more efficiently than crop plants (Chisti, 2007). The oil content of algal species depend on biomass which has three main components *viz.*, carbohydrate, protein and lipid (natural oils). Few algae species can produce upto 60 per cent of their body weight in the form of TAGs (Metting, 1996)The average lipid content among different algae varies between 1 and 70 per cent but under certain conditions some species can reach 90 per cent

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of dry weight (Li *et al.,* 2008). In certain cases oil content in microalgae can reach 75 per cent by weight of dry biomass but associated with low productivities (e.g. for *Botryococcus braunii*). Most common algae (*Chlorella, Crypthecodinium, Cylindrotheca, Dunaliella, Isochrysis, Phormidium Nannochloris, Nannochloropsis, Neochloris, Nitzschia, Phaeodactylum, Porphyridium, Schizochytrium, Tetraselmi*s) have oil levels between 20 and 50 per cent but higher productivities can be reached by using different growth aspect. At present Chlorella seems to be a good option for biofuel production yet there are possibilities with other species like *Lyngbya* sp., *Synechococcus* sp. (Selvan *et al.,* 2011) *Euglena* sp., *Spirogyra* and *Phormidium* sp. (Ramachandra *et al.,* 2013) for having potential of biofuel production.

Phormidium sp. is thermophilc, filamentous, non-heterocyst, blue green algae (Cyanobacteria) belongs to Phormidiaceae family. it can grow in variety of habitat as fresh water, sewage water, waste water and rice field. it's been recognized as rich source of structurally novel and biologically active metabolite generally accumulated in its biomass and can be a part of carotenoid antimicrobial activity (Williamson *et al.,* 2002). This specie may have potential to use as bio fertilizer and in production of biofuels from its lipid content which depends on various factors including ecological and physiological environmental conditions (Ramachandra *et al.,* 2013). The production of biomass may triggered by several influencing factors of photosynthesis. The main factors affecting the photosynthetic productivity of algae include light intensity, temperature, photosynthetic rate, transpiration rate, nutrient availability and lipid production (Abrahim *et al.,* 2013). The nutrient contents such as nitrogen (sodium nitrate), carbon and phosphorus sources of culture medium have great impact on growth of *Phormidium* sp. Sodium nitrate is being used as nitrogen source in several studies (Arumugam *et al*., 2013). Nitrogen is one of the important nutrient for biomass growth and available for plant in for of nitrate. Sodium nitrate is easily available and economically low cost nitrogen source and can be utilized in large scale culturing of algae being commercially available with high lipid content. (Arumugam *et al.,* 2013; Yadavalli *et al.,* 2012); Nitrogen source is also vital for microalgae cell physiology and growth. It is an essential component that contribute to the biomass formed lack of nitrogen will cause chlorophyll reduction and increase in carotenoid which lead to discolouration of cell (Virthie *et al.,* 2010). Thus, present study was conducted with an objective to evaluate the effect of nitrogen in biomass production and lipid accumulation providing through sodium nitrate with BG11 growth medium.

MATERIAL AND METHODS

The experiment was conducted in the research laboratory of Department of Biological Science in SHIATS (Deemed to be University). The growth of *Phormidium* sp. was investigated in liquid BG11 media enriched with different concentration of sodium nitrate for 21 days. The growth rate and biomass productivity has been calculated with the help of formula at 5 days interval and evaluated for best treatment. The cultures was grown in BG-11 medium prepared according to laboratory manual of biofuel containing the following constituents (g/l distilled water): 0.001 *EDTA* disodium salt ; 0.04 K_2HPO_4; 0.075 $MgSO_4 \cdot 7H_2O$; 0.02 $NaCO_3$; 0.036$CaCl_2 \cdot 2H_2O$; 0.006 citric acid; 0.006 ferric ammonium citrate and 1 ml trace elements under 24 h continuous light, 28±1°C, 3000 lux light intensity, at pH 7. Once the desirable amount of *Phormidium* obtained then experiment was carried out by transferring at into different concentration on $NaNO_3$ enrich media. Detailed of treatment are given in Table A. All the experiment work conducted in triplicate.

Table A : Treatment details

Sr. No.	Treatments code	Concentrations of sodium nitrate (g/lit.)
1.	T_0	Control
2.	T_1	0.25
3.	T_2	0.50
4.	T_3	1.00
5.	T_4	1.50
6.	T_5	2.00
7.	T_6	2.50
8.	T_7	3.00
9.	T_8	3.50
10.	T_9	4.00

Growth determination of *Phormidium* sp. :

The growth of algae was estimated by using spectrophotometer. Throw absorbance at a wavelength of 680nm growth was calculated using formula given below (Lee *et al.,* 1998).

Growth of biomass (g/lit)= $0.939XA_{680} + 0.011$

Growth rate (%) biomass of *Phormidium* sp.

The growth rate(%) was determined at particular day using the formula :

$$\text{Growth rate (\%)} = \frac{\text{Final concentration} - \text{Initial concentration}}{\text{Initial concentration}} \times 100$$

TAG's estimation :

TAG estimation was done by Bligh and Dyer (1959). Method 0.5 mg of *Phormidium* was mixed with chloroform – methanol (1:1 Vol/Vol/). Then equivalent vol. of distilled water was add to the *Phormidium* and chloroform – methanol mixture. The mixture were transferred into a separating funnel and agitated for 5 minute. After 5 minute three layers were formed, upper layer lipid, second layer chloroform – methanol mixture and third layer crude oil. The lower layer and middle layer was removed first then TAG (lipid) layer was isolated in test tube and allowed to settle down for time being. Later TAGs was collected in another test tube by using pipette and three times 80 per cent methanol (1.5 ml), diethyl ether (1:50) and catalyst potassium hydroxide (1% KOH or $3HOCH_3$) was added to it. Then finally it was placed in hot air oven at 65^0C for 90 min so that methyl ester fatty acid and glycerol was obtained in 3:1 ratio.

RESULTS AND DISCUSSION

Present study was designed to observe the effect of nitrogen source on biomass production and lipid content in *Phormidium* sp. Sodium nitrate was used as nitrogen source at different concentrations (0.25-4.0g/l) in BG-11 media. Details of biomass production, growth rate and TAG accumulation has been discussed in sub-heads.

Growth evaluation :

Nitrogen was quantitatively most important nutrient affecting the biomass growth and lipid productivity of various microalgae (Griffiths and Harrison, 2009). It is also vital for microalgae cell physiology as lack of nitrogen cause chlorophyll reduction and increase in carotenoids (Virthie *et al.*, 2010), further $NaNO_3$ is easily available and economically low cost nitrogen source having potential to utilize as nitrogen source for biomass development and lipid accumulation in *Phormidium.* Present study reveals that in comparison to control all the treatments showed better performances (Fig.1), however, treatment T_4 (1.5 g/lit.) produced maximum biomass at each interval of 5 days (53.3 mg/lit. at 5^{th} day, 89.9 mg/lit after 10 days, 140.6 mg/lit after 15 days and 135.9 mg/lit after 20 days) followed by treatment of (T_5) 2.0 g/lit treatment where biomass production

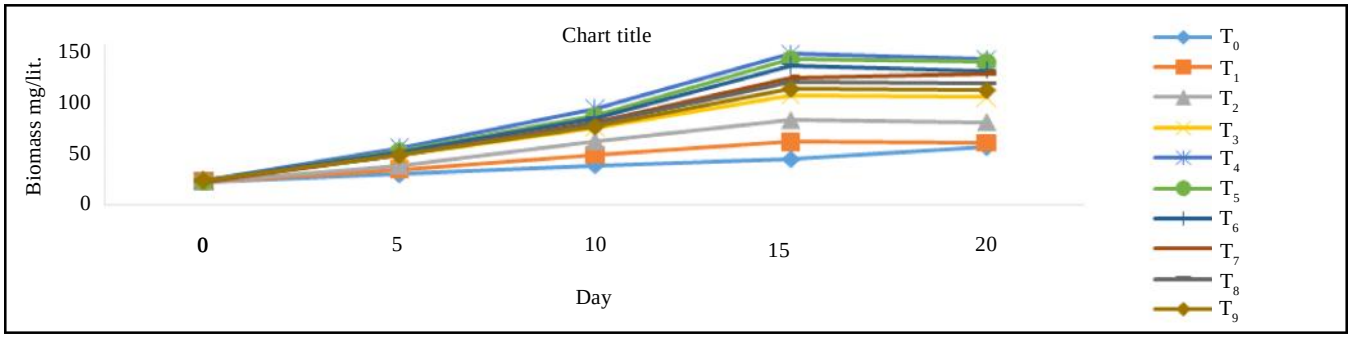

Fig. 1 : Growth of biomass (mg/lit) upto 20 days under different concentration of sodium nitrate

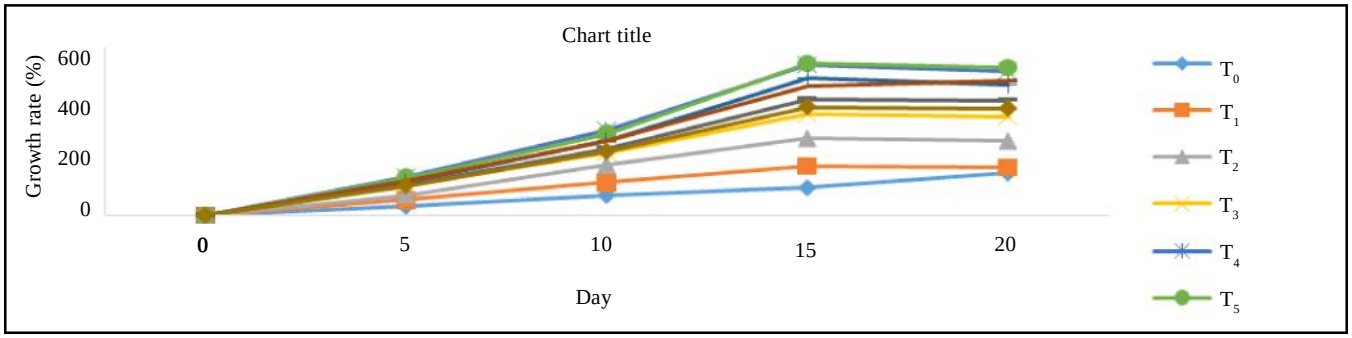

Fig. 2 : Growth rate of biomass (%) upto 20 days under different concentration of sodium nitrate

series was 50.4 mg/lit (5th day), 83.3 mg/lit (10th day), 135.9 mg/lit, (15th day) 133.1 mg/lit, (20th day). Minimum biomass production was obtained with the treatment (T_1) 0.25g/l 33.5 mg/lit (5th day) 46.7 mg/lit (10th day), 58.9 mg/lit (15th day) 58.0 mg/lit (20th day).

Similarly growth rates was maximum (Fig.2) in T_4 for all the measured days *viz.*, 131.54 (5th day), 290.77 (10th day) 511.23 (15th day), 490.81 (20th day) followed by T_5 where growth rate series was 119.30 per cent (5th day), 262.19 per cent (10th day), 490.81 per cent, (15th day) 478.57 per cent, (20th day). Minimum growth rate (T_1) 52.44 per cent (5th day), 112.19 per cent (10th day), 167.68 per cent (15th day), 163.41 per cent (20th day).

Biomass productivity under sodium nitrate enriched BG11 media for *Phormidium* exhibited an increment in growth rate and biomass production at lower concentration of sodium nitrate under 21 days. Biomass production was almost triple from the original concentration under different treatments. Maximum growth was found at lower concentration (1.5 g/lit) of sodium nitrate enriched media which suggest that lower concentration of nitrates gives the good biomass production for algae. Similarly work on *Chlorella pyrenoidosa* cultivated in BG-11 medium with $NaNO_3$ (1.5 g/lit) give maximum biomass production. Further below this concentration (1.5 to 1.2, 0.9, 0.6, 0.3, 0. g/lit) there was linear decrease in biomass production (Sharma *et al.*, 2014). It is also reported that under different source of nitrogen (NH_4C_l, NH_4NO_3 Urea, $CaNO_3$ KNO_3 $NaNO_3$), $NaNO_3$ was proven best in providing nitrogen to developing microalga (Arumugam *et al.*, 2013). *Phormidium* sp. showed the maximum biomass growth at (1.5g/lit) of sodium nitrate (Salem *et al.*, 2013). Microalgae *Botryococcus braunii*, *Scenedesmus dimorphus* (SD7) *Botryococcus* sp. (B6) and *Neochloris oleoabundans* grown in 8.5 mM L^{-1}, 17 mM L^{-1} and 34 mM L^{-1} concentrations of sodium nitrate separately. 8.5 mM L^{-1} concentration showed the maximum biomass production (Rani *et al.*, 2011). And *S. Platensis* grown for 12 days cultures maximum

Table 1: TAG's and MEFA accumulation of different treatment after 20 days

Treatments	TAGs (ml /mg)	MEFA (ml/mg)
T_0	1.1	4.4
T_1	4.3	17.2
T_2	3.9	15.6
T_3	3.5	14
T_4	3.1	12.4
T_5	2.8	11.2
T_6	2.5	10
T_7	2.1	8.4
T_8	1.8	7.2
T_9	1.4	5.6

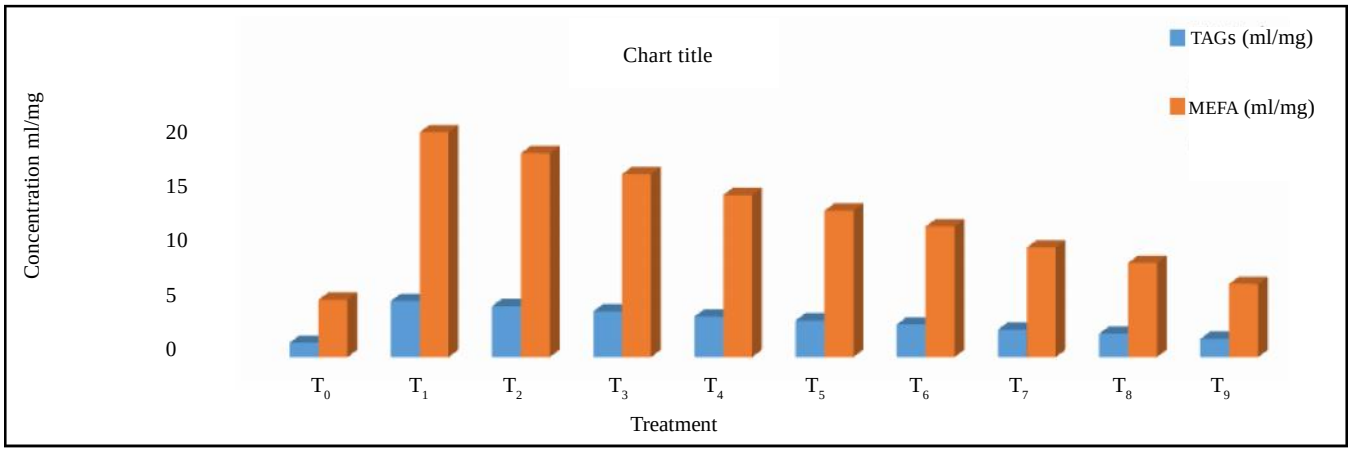

Fig. 3: Estimation of TAG's after 20 days under different concentration of sodium nitrate

biomass production (3.186 g/lit) at 415 ppm of nitrogen source (Abd-El-Baky *et al.*, 2013). The DCW of *C. pyrenoidosa* found to be maximum when sodium nitrate was used as nitrogen source, maximum dry cell weight (DCW) of 2.846 g/lit was obtained in HN-8 medium at 135 µmol $m^{-2}s^{-1}$ (Yadavalli *et al.*, 2012). All this suggest that $NaNO_3$ may be utilized as a source of nitrogen for increase in biomass production for *Phormidium* species but only at low concentration. High concentration may have adverse effect.

The maximum amount of TAG and methyl ester was obtained in treatment T_1 (0.25 g/lit). Its TAG value was 4.3 ml and MEFA was 17.2 ml (Table 2 and Fig.3). The result shows that microalgae enriched with 0.25 g/lit concentration was the most significant treatment for the TAG and MEFA production (Table 1 and Fig. 3). Similarly work on nitrogen deficiency of *C. vulgaris* of 0.1 mM of $NaNO_3$ in the highest lipid content was found (Battah *et al.*, 2013). Its also reported that maximum lipid productivity of *Chlorella pyrenoidosa* at lower nitrogen concentrations was found 0.103 g/d/lit at 135 µmol (Yadavalli *et al.*, 2012). *Synechococcus* sp., *Cyanobacterium aponinum.*, *Phormidium* sp. that the maximum lipid content could be achieved in the medium containing 0.25g/lit $NaNO_3$ (Karatay and Dönmez, 2011). *Neochloris oleoabundans* shows that the highest lipid cell content of 0.40 g/lit was obtained at the lowest sodium nitrate concentration (3 mM), (Li *et al.*, 2008). The lipid content of *Hapalosiphon* sp. cultivated in medium containing 1,500 mg/lit sodium nitrate was 15.78 ± 0.51 per cent. Indeed, the highest lipid yield of 0.063 g/lit was found in a medium containing 375 mg/lit sodium nitrate (Ruangsomboon, 2014). This states that although nitrogen in among one of the growth nutrient but required in low quantity for the biomass production in case of algae further at low concentration it creates nutritional deficiency which brought plant under stress condition which switches its metabolic pathway towards lipid composition from free fatty acid-rich lipid to lipid mostly contained TAG (Widjaja *et al*., 2009). Thus, high lipid concentration obtained although biomass production slowed down due to nitrogen starvation.

Conclusion :

It may be concluded that nitrogen is necessary for the biomass production and low dose of $NANO_3$ (1.5g/lit) can be efficiently use for increase in biomass production for *Phormidium* sp. However, very low concentration (0.25 g/lit) provided better TAG accumulation by provoking stress related reaction.

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