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OPTIMIZATION OF PH AND NITROGEN SOURCE FOR ENHANCED PRODUCTION OF LIPID IN CYANOBACTERIUM *SYNECHOCOCCUS ELONGATUS* PCC7942

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ABSTRACT

Cyanobacteria commonly known as "bluegreen algae" belongs to the kingdom Monera, division cyanophyta are photosynthetic prokaryotes. They are cosmopolitan and found both in fresh and marine water bodies and play a major role in the nitrogen, carbon, and oxygen dynamics of many aquatic environments. Cyanobacteria being important component of biodiversity also, recognized as a new microorganism for isolation of various bioactive compounds with potent nutritional, antibacterial, antioxidant, antifungal, anticancerous, antiviral activities. At present, cyanobacteria have gained much attraction as a feedstock for biofuel production due to their natural advantage to produce lipids. The study deals with the optimization of media component (nitrogen source) and pH condition for enhancement of the lipid content in microalgae. The cyanobacterium *Synechococcus elongatus* PCC7942 was grown in B11 media under controlled environment with altered pH (5 to 9) and nitrogen sources. The result revealed that *Synechococcus* could tolerate the wide range of pH from acidic to alkaline. However, acidic pH significantly retarded the growth of *Synechococcus elongatus* PCC7942. In contrast culture grown in alkaline condition (pH-9) had shown better growth along with higher percentage content of total lipid. In context to nitrogen source, potassium nitrate at concentration 1 g/L was found to be the preferred nitrogen source for optimum growth of *Synechococcus elongatus* PCC7942 with appreciable

amount of total lipid content. Moreover, the maximal percentage of lipid was seen in case of ammonium nitrate at concentration 1 g/L during late stationary phase which might be due to the accumulation of neutral lipid mainly triglycerides.

Key words: Cyanobacteria, *Synechococcus elongatus* PCC7942, lipid content, nitrogen source

INTRODUCTION

Cyanobacteria are sunlight-driven cell factories that convert carbon dioxide into potential biofuels, foods, feed, and high-value bioactive compounds. Few species of cyanobacteria such as *Anabaena, Nostoc* and *Spirulina* are consumed as food due to their high protein and fiber content [1, 2] also, they are rich in vitamins and amino acids [3] and contain significant quantity of lipids and fatty acids. Some microalgae species have the potential to accumulate lipids at more than 50% of their biomass. Total lipids comprises of pigments, phospholipids, glycolipids, and the neutral lipids. Neutral lipids are comprised of triglycerides, free fatty acids, hydrocarbons, sterols, wax and sterol esters and free alcohols. The major lipids present in cyanobacteria being monogalactosyl diacylglycerols, digalactosyl diacylglycerols, sulfoquinovosyl diacylglycerols and, to a minor degree, phosphotidyl glycerol [4].

Recently, cyanobacteria have gained much attraction as a feedstock for biodiesel production due to their natural advantage to produce lipids in high-speed growth. Apart from greater lipid content, cyanobacteria also offer other features such as the shorter period of doubling its biomass than other photosynthetic microorganisms, continuous crop, homogeneous physical structure, and the lack of need for arable. The production of lipid in cyanobacteria greatly affected by parameters such as light quality and quantity, pH, salinity, temperature, and macronutrients, mainly nitrogen (N) and phosphorus (P). Therefore, ideal conditions are prerequisite to produce lipid at the highest productivity. Therefore, the present research aimed to optimize the pH and nitrogen source for enhancement of the lipid content in fresh water cyanobacterium *Synechococcus elongatus* PCC7942.

MATERIAL AND METHODS

Test organisms

The test cultures of non-heterocystous cyanobacterium *Synechococcus elongatus* PCC 7942 was obtained from the University of Allahabad, Uttar Pradesh, India.

Maintenance of stock cultures

2-3 mL of a 3 weeks old cyanobacterial stock culture was used as inoculum in 50 mL of autoclaved BG-11 media in 150 mL

Erlenmeyer flasks. These samples were maintained at 26 ± 2 ^{0}C under white fluorescent light tubes providing an intensity of 75 µmole photons$\cdot m^{-2}\cdot s^{-1}$ and a photoperiod of 14:10 hours. The stock cultures were maintained for 20-30 days.

Optimization of pH of culture media for enhancement of total lipid

For optimization of pH, culture media with different levels of pH (5-9) were used keeping other components of BG-11 media and environmental conditions constant. All the experimental set ups were prepared in 100ml BG-11 media. The *p*H of the experimental media was adjusted to 5, 6, 7, 8 and 9 with the help of 1 N NaOH and 0.5 N HCl. The cultures were maintained for 30 days of study period.

Optimization of nitrogen source for enhanced production of total lipid

For optimization of nitrogen source three nitrate sources were selected *viz*., KNO_3, $NaNO_3$ and NH_4NO_3. *Synechococcus elongatus* PCC 7942 was cultured in BG-11 media of pH-9 while other media components was kept constant.

Growth analysis of experimental organisms in terms of Chlorophyll a content:

Chlorophyll a was estimated by the method of McKinney [5]. After 5th day of inoculation, cells were harvested and were pelleted down by centrifugation at 5000 rpm for 10 min at 4ºC. Supernatant was discarded. Chlorophyll a was extracted in 3ml of 100% chilled methanol through freezing and thawing of samples over night recentrifuged for 10 min at 4°C. For complete extraction of the pigments, the tubes were kept in a refrigerator for 24 h. After the extraction period, the samples were centrifuged and the supernatant was collected. The absorbance of the supernatant was recorded at 680nm against methanol serving as blank. This process was repeated on day 5, 10, 15, 20, 25 and 30 in triplicates.

Chlorophyll a was calculated using the equation: A=Kcl (Where: A-absorbance at 680nm (A680), K- Molar extraction coefficient, which is constant (13.42), C-Concentration (mg/ml) and L - Path length which is usually 1).

Biomass harvest

The biomass was harvested by filtration and then concentrated by centrifugation at 15,000 rpm for 15 min. The cell pellets were suspended in deionized water and re-centrifuged two times to remove residual medium.

Extraction of lipid

Total lipid from *Synechococcus elongatus* PCC 7942 was extracted according to the Bligh and Dyer standard method with some modifications [6]. Briefly, methanol–chloroform 2:1 (v/v) was added to extract the lipids from the cells of *Synechococcus*

elongatus PCC 7942, the mixture was sonicated for 15 min, and then centrifuged and the supernatant was transferred by means of a pipette to another tube. The precipitated residual material was then re-suspended in methanol–chloroform–water 2:1:0.8 (v/v/v) and the mixture were again sonicated for 15 minutes. The phase separation was obtained by centrifugation, and the lower chloroform phase was withdrawn. The combined chloroform phases were dried in a rotatory evaporator and the lipids and pigments obtained were re-dissolved in chloroform and stored at -20 °C.

Quantification of total lipid content

Total lipids were quantified by gravimetric method. The weight of the crude lipid obtained from each sample was measured by following equation.

Weight of lipid = (weight of container + extracted lipid) – (weight of container)

Lipid content (%) = {amount of lipid extracted (g)} ×100/{weight of original sample (g)}

Characterization of extracted lipid by thin layer chromatography

Thin layer chromatography was carried out on plates of size 20cm X 20 cm using prepared silica plate from MERCK. Plates were activated at 120^{o}C for 2h in hot air oven. After the application of extracts, the plates were developed in the chromatographic tank saturated with the solvent. The solvent system used was chloroform: methanol: 25% ammonia solution (65:25:4) [7].

Plates were exposed to iodine vapor in an air tight tank in fume hood. All unsaturated and saturated lipids appeared as brown spots [8].

Determination of R_f values

The distance of each band migrated was measured and the R_f value was calculated in relation to the solvent front by the following formula:-

R_f value = (Distance travelled by sample)/ (Distance travelled by solvent)

RESULTS AND DISCUSSION

Growth behavior of *Synechococcus elongatus* PCC 7942 during pH optimization

The growth behavior was observed in terms of chlorophyll-a content. The pattern of growth exhibited correlation with increasing pH between 5 to 9, the maximal growth was observed in media with pH 9 in *Synechococcus elongatus* PCC 7942 (figure 1). However, it was also observed that model organism were also capable to tolerate acidic media (pH 5 and 6). In the study increase in chlorophyll ‘a’ content was observed from day 5 to day 20, after day 20 there was a sharp decline in chlorophyll a content was observed. Decrease in chlorophyll ‘a’ content after day 20, might be due the reason that cells have reached in stationary phase which

resulted in reduced photosynthesis and reduced growth.

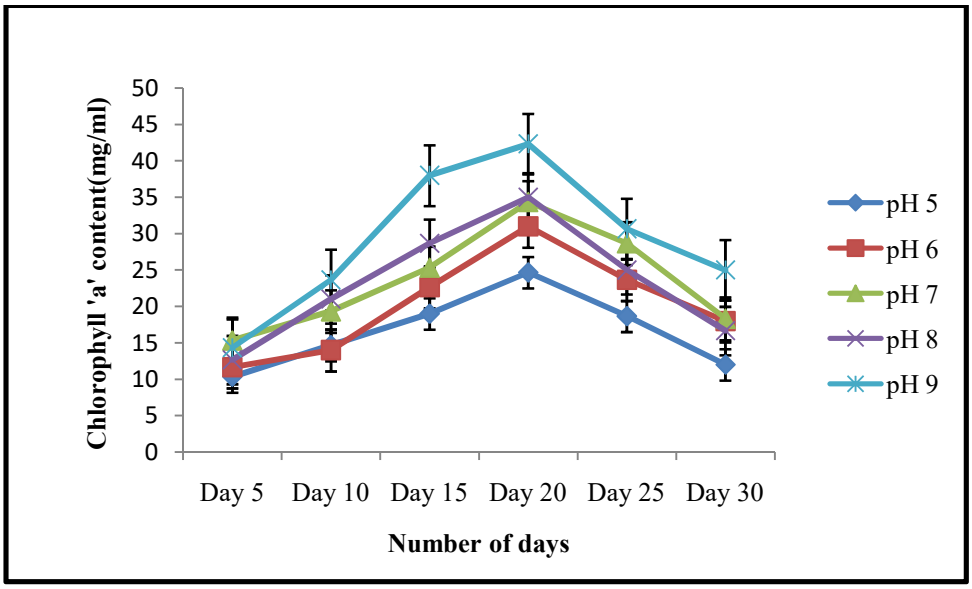

Figure 1: Growth curve of *Synechococcus elongatus* PCC 7942 in terms of chlorophyll 'a' content

Estimation of total percentage content at variable pH

After growth optimization cells were harvested in early stationary phase i.e. on day 25 because it was revealed from earlier reports that with the progression of stationary phase, accumulation of neutral lipids in the form of triglycerides were increased rather than polar lipids located in the cell membrane [9]. On day 25 the cells were harvested from different experimental cultures (pH 5 to 9) and the lipids were extracted by Bligh and Dyer method and percentage of lipid content were determined gravimetrically (Table 1). It was observed that maximum percentage lipid content was obtained at pH-9. However compared to the control pH (pH 7) there was a slight decline of 2% was observed pH-8 followed by increase of 6% at pH-9. Variation in pH can affect algal growth in a number of ways. It can change the distribution of carbon dioxide species and carbon availability, alter the availability of trace metals and essential nutrients, and at extreme pH levels potentially cause direct physiological effects [10]. Moreover, this study was also in agreement with study conducted by various researchers [11, 12, 13] demonstrating pH between 7.4 and 8.0 were favorable for the optimum growth of cyanobacterial species. There were also many reports demonstrating acidic conditions for growth of cyanobacteria which indicates that cyanobacteria can adapt to variable pH conditions for their growth [14, 15].

Table 1: Total percentage content of lipid during pH optimization

Different pH	Percentage of lipid content in terms of fresh wt of cyanobacteria *Synechococcus* PCC7942		
	Exponential phase	Early Stationary phase	Stationary phase
pH 5	18	18	19.75
pH 6	16.75	17	18
pH 7	13	13	13.65
pH 8	15	15	17.25
pH 9	19	19	21

Preliminary identification of lipid classes at different pH by TLC

Extracted lipid was preliminary identified by thin layer chromatography (TLC). The TLC chromatogram of *Synechococcus elongatus* PCC 7942 exhibited the presence of lipid bands of different Rf values. The TLC chromatogram result had shown the presence of glycolipids, neutral lipids and phospholipids. The culture with pH-5 has shown the presence of three bands of lipid, in which two might be of neutral lipid (Triacylglycerols and Sterol esters) and one of glycolipids (Monogalactosyl diacylglycerol). The culture with pH-6 has exhibited the presence of five bands in which two bands might be of glycolipids (Monogalactosyl diacylglycerol and Esterified steryl glucoside) and two bands were of neutral lipid (Triacylglycerols and Sterol esters) one band was of phospholipid (Phosphatidylcholine). The culture with pH-7 has revealed the presence of six bands in which three bands were of neutral lipid (Free fatty acids, Triacylglycerols and Sterol esters), two bands were of glycolipids (Esterified steryl glucoside and Monogalactosyldiacylglycerol). The culture with pH-8 shown the presence of five bands in which three bands were of neutral lipid(Free fatty acids, Triacylglycerols and Sterol esters) and one band each of glycolipids (Esterified steryl glucoside) and phospholipid (Phosphatidylcholine) was also seen. The pH-9 have shown the presence of six bands in which four bands were of neutral lipid(Free fatty acids, Triacylglycerols, Digalactosyldiacylglycerol and Sterol esters) and one band glycolipids (Esterified steryl glucoside) and two bands phospholipid (Phosphatidylcholine and Phosphatidylethanolamine) . Rf value of different bands are presented in table 2.

Table 2: Preliminary identification of lipid classes at variable pH by TLC

Lipid class	Rf value	different pH levels				
		5	6	7	8	9
Sulphoquinovosyldiacylglycerol	6	-	-	-	-	-
Phosphatidylinositol (PL)	11	-	-	-	-	-
Digalactosyldiacylglycerol	17	-	-	-	-	+
Phosphatidylcholine (PL)	20	-	+	+	+	+
Phosphatidylethanolamine (PL)	30	-	-	-	-	+
Steryl glucoside	41	-	-	-	-	-
Phosphatidylserine (PL)	47	-	-	-	-	-
Free fatty acids(NL)	56	-	-	+	+	+
Monogalactosyldiacylglycerol	64	±	±	+	-	-
Esterified steryl glucoside	76		+	+	+	+
Triacylglycerols (NL)	79	+	+	+	+	+
Sterol esters	95	+	+	+	+	++

*'-' symbolizes absence brown color; *'+' symbolizes light brown color; * '±' symbolizes pale brown color (**Ramadan M.F., Asker M.M.S., Ibrahim Z.K. (2008).**

Optimization of nitrogen source for enhancement of lipid content of *Synechococcus elongatus* PCC7942

The nitrogen sources selected for the study were potassium nitrate, sodium nitrate and ammonium nitrate. Since nitrogen source was among primary factor influencing growth rate and chemical compositions of cyanobacteria. In this study KNO_3 was proved to be one of the best nitrogen sources in terms of maximum growth. In both the cases with KNO_3 and $NaNO_3$, cultures reached early stationary phase on day 25 revealed by decrease in chlorophyll 'a' content. In contrast, Chlorophyll a content was found lower with ammonium nitrate and cultures reached early stationary phase on day 20 (Figure 2).

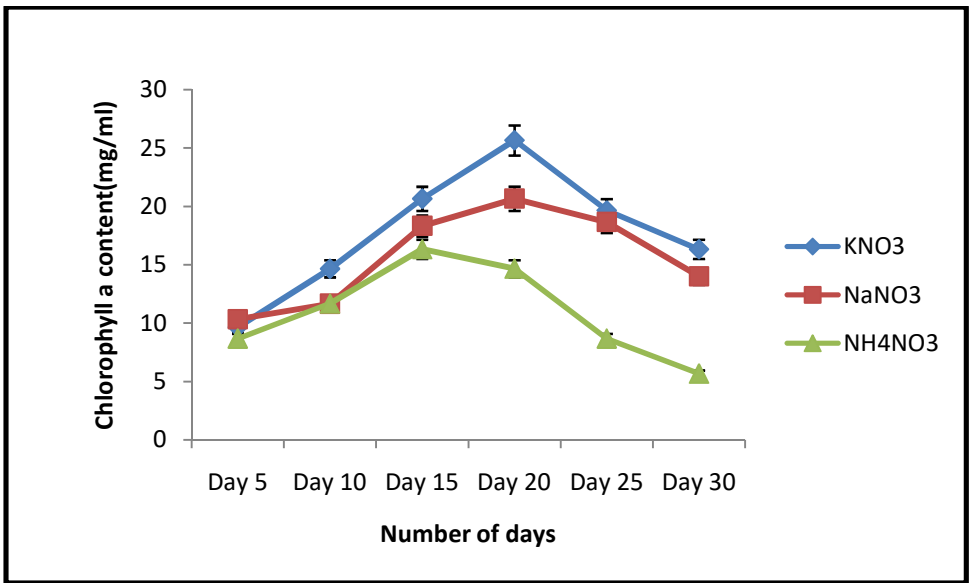

Figure 2: Growth curve of *Synechococcus elongatus* PCC 7942 in terms of chlorophyll 'a' content

Estimation of total percentage content of lipid with different nitrogen source

Nitrogen, one of the most indispensable elements for growing microalgae, is integral to a great number of cellular metabolic activities. Usually, there is positive correlation between cyanobacterium growth and nitrogen concentration and negative correlation between lipid accumulation in microalgal cells and nitrogen concentration in the medium [16, 17]. In present study it was found that percentage content of lipid was maximum in culture incubated with ammonium nitrate, which was slightly higher as compared to KNO_3 followed by $NaNO_3$ (Table 3) which might be due to the increase level of neutral lipids which tend to increase during the stress conditions.

Preliminary identification of lipid classes by TLC during nitrogen source optimization

The TLC chromatogram result shown that there was presence of similar bands as like cultures incubated at pH-9 with KNO_3 and $NaNO_3$. In case of cells incubated with nitrogen source $NaNO_3$, the chromatogram shown the presence of six bands of Rf value 17, 20, 56, 76, 79 and 95 which might be of Free fatty acids, Triacylglycerols, Digalactosyl diacylglycerol and Sterol esters(neutral lipids), one band of esterified steryl glucoside (glycolipids) and one bands of phosphatidylcholine (phospholipid) repectively. However, TLC chromatogragm in case of culture incubated with nitrogen source ammonium nitrate showed the presence of five bands of Rf value 56, 64, 76, 79 and 95. Three of them are more prominent they might be correspond to the lipid classes, free fatty acids, triacylglycerols and sterol esters, all were neutral lipids while other two were monogalactosyldiacylglycerol, and esterified steryl glucoside (glycolipids) (Table 4).

Table 3: Optimization of nitrogen source for optimum growth of *Synechococcus elongatus* PCC7942

Different Nitrogen source	Percentage of lipid content in terms of fresh wt of cyanobacteria *Synechococcus* PCC7942		
	Exponential phase	Early stationary phase	Late stationary phase
KNO_3	12	12.98	14
$NaNO_3$	10.65	11	11.85
NH_4NO_3	13.25	14	15.56

Table 4: Preliminary identification of lipid classes by TLC during nitrogen source optimization

Lipid class		Nitrogen source		
	Rf	KNO_3	$NaNO_3$	NH_4NO_3
Sulphoquinovosyldiacylglycerol	6	-	-	-
Phosphatidylinositol (PL)	11	-	-	-
Digalactosyldiacylglycerol	17	+	+	-
Phosphatidylcholine (PL)	20	+	+	-
Phosphatidylethanolamine (PL)	30	+	-	-
Steryl glucoside	41	-	-	-
Phosphatidylserine (PL)	47	-	-	-
Free fatty acids(NL)	56	+	+	+
Monogalactosyldiacylglycerol	64	-	-	±
Esterified steryl glucoside	76	+	±	±
Triacylglycerols (NL)	79	+	+	+
Sterol esters	95	++	±	+

***'-' symbolizes absence brown color; *'+' symbolizes light brown color; * '±' symbolizes pale brown color* (Ramadan M.F., Asker M.M.S., Ibrahim Z.K. (2008)**

CONCLUSION

For economical production of biofuel from microalgae, biomass as well as lipid content plays an important role**.** In the present study, the results mentioned above indicate both nitrogen source and pH of culture media played an important role in enhancement of total lipid content. From the above study it could be concluded that cyanobacterium *Synechococcus elongatus* PCC7942 can tolerate acidic to alkaline pH. However, for enhanced production of lipid in terms of both optimum growth and good percentage yield of lipid, the cyanobacterium *Synechococcus elongatus* PCC7942 should be cultured in BG-11 media of pH-9 with preferred nitrogen source potassium nitrate.

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