Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Annals of Biological Research, 2015, 6 (11):47-54
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Studies on bioflocculant exopolysaccharides production by *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35

Romi Khangembam[1*], Angom Thadoi Devi[1], Chungkham Silvia[1], Aribam Subhalaxmi Sharma[1], Ojit Singh Keithellakpam[1], Avijeet Singh Oinam[1], Gunapati Oinam[1], Thingujam Indrama[1], Wangkhem Indira Devi[1], Kangjam Sarabati Devi[1], Longjam Miranda[1], Onkar Nath Tiwari[1], Girdhari Lal Tiwari[2] and Mohan Chandra Kalita[3]

[1,1]National Repository for Cyanobacteria and Microgreen algae (Freshwater), Microbial Resources Division, Institute of Bioresources and Sustainable Development, (A National Institute of DBT, Govt. of India), Takyelpat, Imphal, Manipur, India*
[2]Department of Botany, University of Allahabad, Allahabad, U.P., India
[3]Department of Biotechnology, Gauhati University, Guwahati, Assam, India

---

ABSTRACT

Bioflocculant exopolysaccharide (EPS) production by ten (10) cyanobacterial strains during their photoautotrophic growth was investigated. Lyngbya sp. BTA166 and Anabaena sp. BTA35 produced highest levels of EPS along with highest flocculating activity amongst the studied strains. 16S rRNA nucleotide sequencing and BLAST analysis of nucleotide sequences were used to identify the cyanobacterial strains. The sequences were deposited in GenBank and accession numbers were obtained as KJ830952 and KJ562184, respectively. Studies on the effect of incubation period on EPS production revealed that highest EPS production was observed during stationary growth phase and late phase of growth. Biochemical composition of the exopolysaccharides from the two potential strains was also investigated. The cyanobacterial EPS consisted of soluble protein and polysaccharide that included substantial amounts of neutral sugars and uronic acid. The flocculant bound a cationic dye, alcian blue, indicating it to be polyanionic. The results of these experiments indicate that strains Lyngbya sp. BTA166 and Anabaena sp. BTA35 are good candidates for the production of EPS which could be utilized in industrial or commercial applications as an alternative to synthetic and abiotic flocculants.

Keywords: *Anabaena* sp., Exopolysaccharide, Flocculants, *Lyngbya* sp., Uronic acid

---

INTRODUCTION

Flocculants are widely used in various industrial processes such as drinking water treatment, downstream processes, wastewater treatment plants, and in different fermentation processes [1]. Although chemical flocculants have numerous advantages of being effective in terms of flocculating efficiency, affordability and availability, their usage has been reported to be harmful to humans [2]. In recent years, many studies have been undertaken where different microorganisms such as algae, fungi, bacteria and actinomycetes have been used in bioflocculant production [3, 4].
In order to circumvent the health and environmental problems attributed to inorganic and synthetic flocculants, flocculants produced by microorganisms have attracted considerable scientific and technological attention in recent years [5]. The interest in biotechnological methods for the production of bioflocculants lies in the possibility of

using different organisms to synthesize extracellular substances with different compositions [6, 7, 8]. Natural flocculants having a plant or microbial origin, may be composed of polysaccharide, proteins, lipids, lipoproteins and lipopolysaccharide and flocculation by them involves the polymer chain sticking to multiple particles making an aggregate large enough to settle down. Compared to chemical flocculants, bioflocculants have unrivaled benefits because they are environmentally friendly, biodegradable, nontoxic and harmless to human and animal health, and free of the risk of secondary pollution.

Many strains of cyanobacteria produced substantial quantities of exopolysaccharides [9, 10, 11]. It has been suggested that EPS produced by cyanobacteria could be useful in various applications due to their water-holding capacity in soils and their ability to remove heavy metals and solid materials from water reservoirs [12, 13, 14].
As part of our exploration for new and novel bioflocculants that could stand as alternatives to inorganic and synthetic flocculants, the present study reports on the bioflocculant-producing potential of freshwater cyanobacterial strains and determine the influence of different light quality and pH on EPS production, and assess a potential commercial application of cyanobacterial EPS.

MATERIALS AND METHODS

Cyanobacterial strains conditions and microscopic observations

For this study, ten (10) fast-growing cyanobacterial strains were obtained from the National Repository for Cyanobacteria and Microgreen algae (Freshwater) of the Institute of Bioresources and Sustainable Development, Imphal, Manipur, India. All of these strains were originally isolated from North-Eastern region of India. Cultivation was conducted in 250 ml Erlenmeyer flasks containing 100 ml of BG-11 medium [15]. The nitrate component of the medium was excluded for culturing heterocystous strains except when otherwise indicated, but was included for culturing all non-heterocystous strains. Batch cultures were prepared for characterizing EPS production by centrifuging a culture of a desired strain while in exponential growth, then transferring 50 mg of the wet biomass to a flask containing 100 ml of culture medium. Inoculated cultures were grown photoautotrophically at 28±2°C under a light/dark cycle of 14/10 h at a light intensity of 54-67 µmol photon $m^{-2}s^{-1}$. Cultures were left undisturbed during growth except for brief mixing twice daily for sufficient mass transfer of media nutrients to, and secreted metabolites from, the organisms. Aliquots of cultures were negatively stained using Indian ink (Himedia, India) as described by [16] as prior to their microscopic observation, in order to observe EPS. Microscopic observations utilized an Axio Scope A1 microscope coupled with software AxioVision 4.7.2. (Carl Zeiss, Gottingen, Germany).

Exopolysaccharide assay and flocculating activity test

Exopolysaccharides from the cell wall of cyanobacteria were separated by putting two pins in the culture flasks and kept in a magnetic stirrer for 15 min. The cells were macerated and cell wall polysaccharides were made to release in the medium. Then, soluble EPS was separated from intact cyanobacteria by centrifugation of cultures at 6600 X g at 15°C for 20 min (Eppendorf, 5430 R, Germany). The supernatant was concentrated to one-fourth of its original volume by drying in a hot-air oven (Universal Oven - 143, Narang Scientific Works, India) at 60°C for 10-12 h. The concentrated liquid was precipitated by the gradual addition of three volumes of cold ethanol and was then kept at 4ºC overnight [17]. The precipitate was washed twice by suspension in cold ethanol, followed by centrifugation. The gel-like pellet obtained after the final centrifugation was collected and put into a dialysis bag (Dialysis membrane-110, Himedia, India). The dialysis membrane was cut with respect to the quantity of pellet obtained and tied with a thread at both ends and was dialyzed against 5 volumes of distilled water overnight at room temperature. The dialysate was then dried at 60ºC to a constant weight.

The dialyzed EPS from two strains, *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35, was analysed chemically. Total neutral sugar content was estimated by Anthrone method [18], using glucose as a standard. Total soluble protein was measured according to [19] using bovine serum albumin as a standard. EPS was hydrolysed for uronic acid estimation. 1 mg of EPS was dissolved in 1 ml distilled water, added 2.4 ml of sulphuric acid and uronic acid content from the hydrolysed EPS was determined spectrophotometrically using the Carbazole method [20], with galacturonic acid as a standard.

Bioflocculant capacity of the EPS extract was determined by a little modification of Alcian blue binding assay [21]. Alcian blue 8GX (Himedia, India) was dissolved at a concentration of 1 mg ml^{-1} in 0.5 N acetic acid. After culture centrifugation, 0.5 ml of supernatant containing EPS was diluted in 4.25 ml of 0.5 N acetic acid and combined with 0.25 ml of the Alcian blue dye preparation. After 30 min incubation at room temperature, the solution was

centrifuged at 2900 X g for 10 min and the optical density of the supernatant was determined at 610 nm (UV-1800, Shimadzu, Japan). Control assays contained Alcian blue and acetic acid without any added EPS.

Flocculating activity was calculated as:
Flocculating activity = [(B-A)/B] X 100%,
Where A and B are the absorbance values of sample and control respectively at 610 nm.

Time course assay of EPS production
EPS production and biomass production were studied at different growth phases. Experiments were carried out in triplicates to reproduce the experiment and standard deviations were also obtained.

Strain identification and phylogenetic affiliation
Genomic DNA was extracted by a modified Xanthogenate method [22]. PCR amplification of 16S rRNA gene sequences was performed using the universal forward primer 536F and reverse primer 1488R [23]. All PCR reactions were performed in a total volume of 50 µl containing 200 µM dNTPs, 0.3 µM of each primer, 1X Taq buffer, 5 U *Taq* DNA polymerase, and 2 µl of genomic DNA. Amplification was performed in a thermal cycler (Mastercycler gradient, Eppendorf, Germany). After an initial denaturation at 95 °C for 5 min, the mixture was subjected to 28 cycles including final denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and final extension at 72 °C for 1 min. The PCR product was detected with standard agarose gel electrophoresis (Elchrom Scientific GEPS 200/2000, Switzerland), and quantification of PCR product was done with BioSpectrometer (Eppendorf, Germany). Sequences of both the directions were aligned using the Clustal W program [24], and partial 16S rRNA gene sequence was compared with other known gene sequences retrieved from the NCBI GenBank. The identification was authenticated through BLAST search (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). The evolutionary history was inferred using the Maximum Parsimony method [25]. The MP tree was obtained using the Close-Neighbor-Interchange algorithm [26] with search level in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option) and phylogenetic analyses were conducted by using MEGA 4 software [27].

RESULTS

EPS production
10 different cyanobacteria used in this study included 05 heterocystous and 05 non-heterocystous filamentous strains. The strains that produced the maximum EPS, *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35 contained a thick surrounding capsule or slime layer that caused the medium within which they were grown to become highly viscous. Both strains contained heterocysts. Figs. 1 and 2 illustrate the appearance of cyanobacterial filaments in the absence and presence of Indian ink. The dye was excluded from the filaments and their EPS surroundings, but caused some distension of vegetative cells. No EPS was visible in the absence of the Indian ink. The cells appeared slightly larger in the India ink stained cells and the unstained part around the organisms confirmed the production of mucilaginous boundary of negatively charged EPS by the cyanobacterial strains.

EPS characterization and flocculating activity
All ten (10) of the examined cyanobacterial strains were screened for EPS production by separating and recovering large molecules from the supernatant of cultures. *Lyngbya* sp. BTA166 produced the highest level of exopolysaccharide (1.20±0.08 mg ml^{-1}), followed by *Anabaena* sp. BTA35 (1.12±0.06 mg ml^{-1}). The total amount of EPS includes the released polysaccharides in the medium or slime polysaccharides. The total EPS and flocculating activity of the studied strains are indicated in Table 1.

Chemical analysis of the EPS extracted from *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35 showed the presence of significant levels of neutral sugars, total soluble proteins and uronic acid. The biochemical composition of EPS for the two highest producing strains, *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35, was presented (Table 2).

Time course assay of EPS production
Results of growth experiments in BG-11 medium revealed that the organisms grew exponentially to a period of 20 days and then entered the stationary phase. The accumulated quantity of EPS increased during growth over time. The maximum amount was obtained from the cultures on day 28, several days after the organisms had entered

stationary phase growth. EPS production increased linearly for the first 20 days after inoculation, followed by a more rapid increase as the cultures approached stationary phase. The rate of EPS production and flocculant activity with respect to the biomass concentration for *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35 were presented (Figs. 3 and 4).

Strain Identification and Its Phylogeny

The 16S rRNA gene sequences of *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35 were found to have closest proximity with *Lyngbya* sp. HQ419195 and *Anabaena* sp. FM177480 respectively. The 16S rRNA gene sequences were deposited at NCBI GenBank with accession numbers as KJ830952 and KJ562184 for *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35, respectively. In the phylogenetic tree (Fig. 5), the consensus tree inferred from 7 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. There were a total of 209 positions in the final dataset, out of which 209 were parsimony informative. The clustering in the dendrogram was well supported by bootstrap analysis and partly reflected the morphological similarity of the organisms. The distances in the tree created by 16S rRNA gene sequencing indicated the evolutionary relationships between the studied strains and *Lyngbya* sp. and *Anabaena* sp. originating from distinct geographical sites.

DISCUSSION

There is a global need for biodegradable and renewable flocculants to replace the chemical flocculants in widespread use. Although bioflocculants hold great potential for broad real world applications, replacing chemical flocculants with bioflocculants would require significant reduction of their production costs. To this end, we set out to screen and isolate novel bioflocculant producers from different freshwater cyanobacterial strains. In this study, we have characterized strains *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35 for its growth and favourable conditions for production of its bioflocculant.

Microscopic observations of negatively stained cells showed the presence of a thick sheath or slime layer surrounding the cyanobacteria examined in this study. The mucilage produced by the organisms prevented the staining of the negative stain, India ink, indicating the production of negatively charged EPS. The presence of non-capsulated morphotypes of cyanobacteria is of crucial importance for the development of biotechnological applications of cyanobacteria since the presence of thick polysaccharidic investments imposes great difficulties for the extraction of bioactive substances and for the molecular biology studies of these strains [28].

In the strains of *Cyanothece*, *Nostoc calcicola* and *Anabaena* were reported to produce 1.77 mg ml^{-1}, 0.7 mg ml^{-1} and 0.055 mg ml^{-1} of EPS, respectively [29, 30, 31]. The values observed in the present study were among the highest reported for any EPS-producing cyanobacterium or EPS-producing lactic acid bacteria [32]. The strains utilized in this study produced large amounts of EPS relative to the amounts reported for other cyanobacteria, reaching 1.78 mg ml^{-1} of culture. *Oscillatoria formosa* was reported to produce 334.8 µg ml^{-1} in 24 days [33].

The highest flocculating activity observed in this study utilized EPS isolated from *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35. Maximum EPS production occurred in the cultures in stationary and late phase. Similar results were also reported [34, 35, 36]. It was studied earlier that stage and age of cultures affect the production of cyanobacterial intracellular polymeric substances [37] which can be correlate with our present findings. On the other hand, entirely dissociated kinetics of cellular growth and rate of EPS production in *Arthrospira platensis* was also demonstrated [38].

A chemical analysis of the two strains with highest EPS production demonstrated the presence of neutral sugars, soluble proteins and uronic acid. Analysis of EPS production by the unicellular cyanobacterium, *Cyanothece* sp. isolated from a rice field of Vietnam, demonstrated a considerable quantity of EPS that contained various sugars and uronic acid [39]. The presence of uronic acid imparts anionic character to EPS, and the capacity of charged groups to bind water molecules can be exploited by the cosmetic industry for product formulations [40]. In recent years, the heavy metal chelating ability of cyanobacterial EPS has been widely exploited for treatment of wastewater [41].
The flocculating activity of MMF1 isolated from the screening medium was 82.9% [42], which can be compared with our present studied strains. For the industrial applications of p-KG03, as the bioflocculant agent, p-KG03 showed that more than 90% of the flocculating activity in kaolin suspension occurred at concentrations of 0.5 mg l^{-1} with the maximum at 1.0 mg l^{-1} [9]. Since the anionic density of the EPS is attributed to carboxyl groups, Alcian

Blue, a cationic dye with a high affinity for polyanions was measured [43], to determine the application of bioflocculant. In the present study, cyanobacterial EPS containing a high uronic acid content exhibited high flocculating activity.

Table 1: Total EPS production and flocculating activity of the ten EPS-producing strains

SN	Name of the strains	EPS production (mg/ml)	Bioflocculant activity
1	*Lyngbya* sp. BTA166	1.20±0.08	81.04%
2	*Anabaena* sp. BTA35	1.12±0.06	78.02%
3	*Anabaena oryzae* BTA109	1.05±0.09	62.08%
4	*Lyngbya* sp. BTA193	0.99±0.06	58.70%
5	*Oscillatoria* sp. BTA291	0.90±0.04	43.07%
6	*Anabaena fertilissima* BTA30	0.92±0.07	45.09%
7	*Phormidium fragile* BTA02	0.89±0.04	38.43%
8	*Phormidium tenue* BTA225	0.87±0.09	46%
9	*Dichothrix* sp.BTA833	0.08±0.08	18.3%
10	*Microchaete* sp. BTA946	0.60±0.02	23.21%

Table 2: Biochemical composition of EPS in the two strains with highest EPS productivity.

Cyanobacteria	Total neutral sugar (µg mg^{-1} of EPS)	Total soluble proteins (µg mg^{-1} of EPS)	Uronic Acid (µg mg^{-1} of EPS)
Lyngbya sp. BTA166	145.40±3.07	67.42±2.01	201.52±6.80
Anabaena sp. BTA35	106.37±5.26	44.96±3.05	183.6±7.50

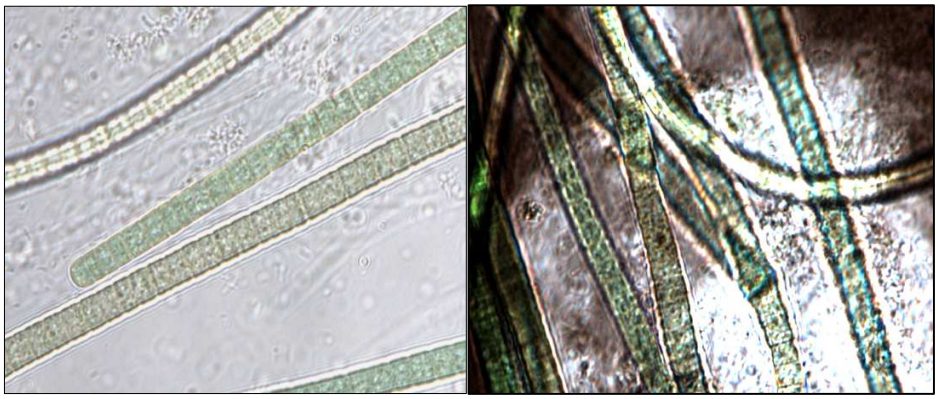

Fig. 1: Brightfield microscope images of *Lyngbya* sp. BTA166 and in the absence (left panel) and presence (right panel) of Indian ink.

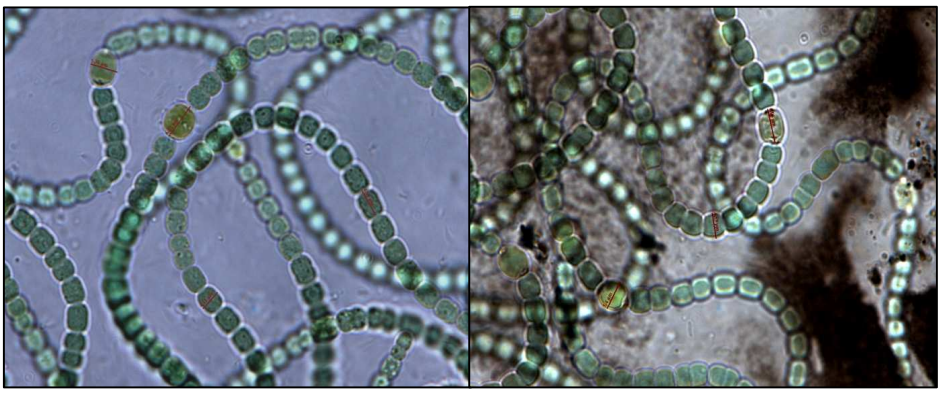

Fig. 2: Brightfield microscope images of *Anabaena* sp. BTA35 in the absence (left panel) and presence (right panel) of Indian ink.

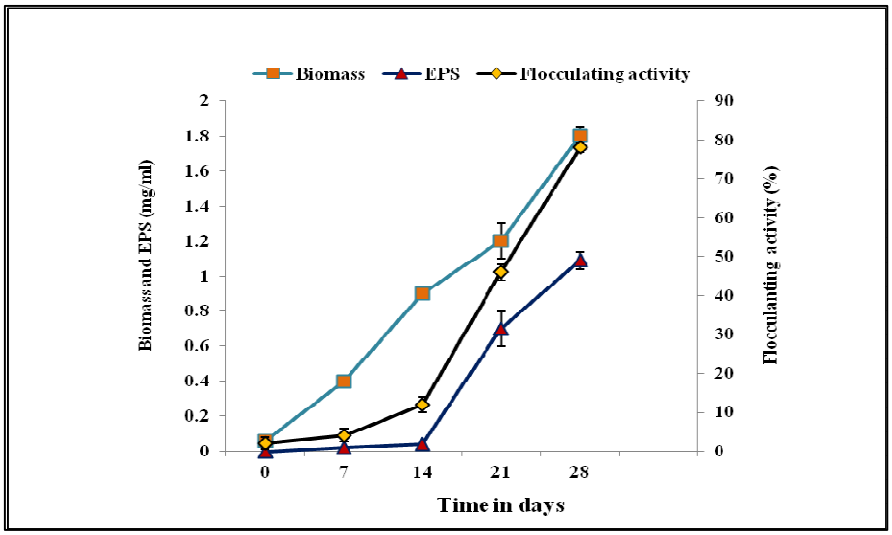

Fig. 3: Extracellular polysaccharide production and bioflocculant activity with respect to biomass production of *Lyngbya* sp. BTA166.

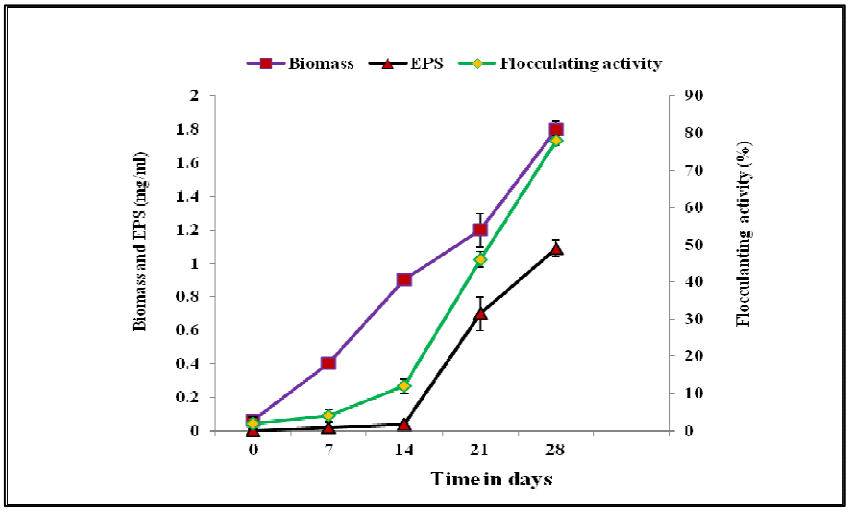

Fig. 4: Extracellular polysaccharide production and bioflocculant activity with respect to biomass production of *Anabaena* sp. BTA35.

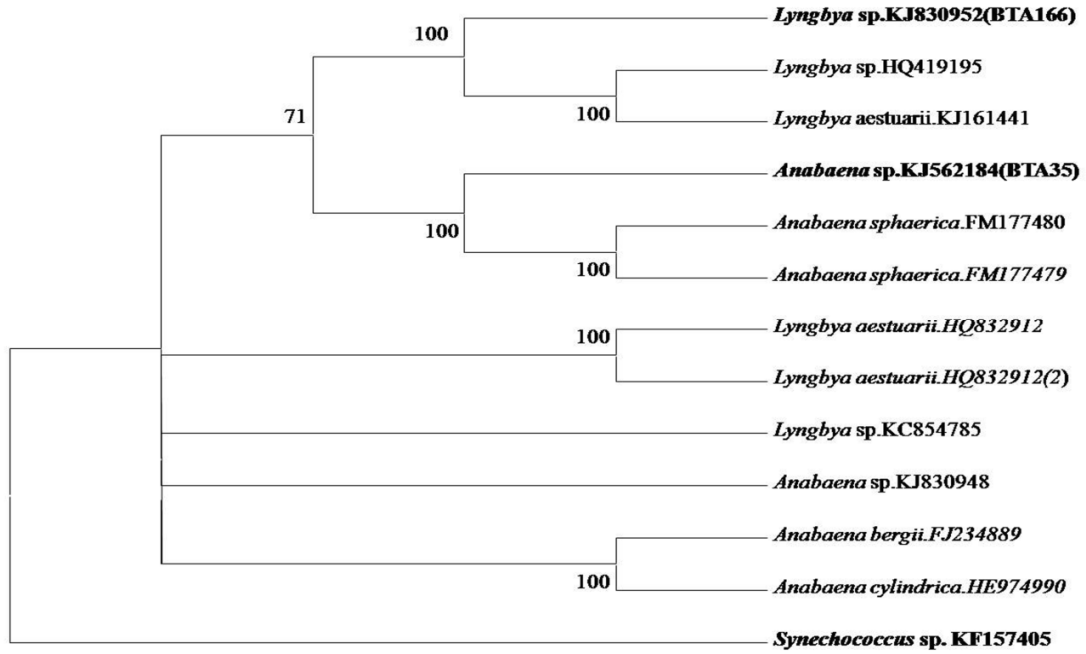

Fig. 5: Maximum parsimomy (Kimura) tree based on partial 16S rRNA gene sequence. The tree includes sequences of Lyngbya sp. BTA166 and Anabaena sp. BTA35 determined in the present study (bold) with their respective NCBI accession numbers KJ830952 and KJ562184 along with 10 sequences from NCBI GenBank database. Synechococcus sp. was used as an out-group.

CONCLUSION

The bioflocculant produced by *Lyngbya* sp. BTA166 and *Anabaena* sp. BTA35 is composed of proteins and polysaccharides and probably other constituents which have contributed to the high flocculation of alcian blue from the solution. In addition, the two strains have shown good EPS producing potential, following high flocculation activity and bioflocculant yield obtained in comparison to the yield and flocculation activity shown by the other reported cultures. Hence, bioflocculant produced by these two strains have good potentials for industrial applications.

Acknowledgements
Authors are thankful to the Director, IBSD, Imphal, Manipur, India for providing laboratory facilities and Department of Biotechnology, Government of India for financial assistance vide grant no: BT/218/NE/TBP/2011.

REFERENCES

[1] IL Shih; YT Van; LC Yeh; HG Lin, YN Chang. *Bioresour. Technol*., **2001**, 78, 267-272.
[2] M Vanhoric; W Mones. Carcinogen of acrylamide. *Carcinogensis*, **1983**, 4, 1459-1463.
[3] SQ Xia; ZQ Zhan; XJ, Wang; AM Yang; L Chen; JF Zhao; L Didier; JR Nicole. *Bioresour. Technol*., **2008**, 99, 6520-6527.
[4] SB Deng; RB Bai; XM Hu; Q Luo. *Appl. Microbiol. Biotechnol*., **2003**, 60, 588-593.
[5] Z Li; S Zhong; HY Lei; RW Chen; Q Yu; HL Li. *Bioresour. Technol*., **2009**, 100, 3650-3656.
[6] M Taniguchi; K Kato; O Matsui; X Ping; H Nakayama; Y Usuki. *J. Biosci. Bioeng.*, **2005**, 100, 207-211.
[7] XY Gong, ZK Luan, YSSG Pei. *J Environ. Sci. Health.*, **2003,** 38, 657-669.
[8] Sahar Z; E Marwa; E Marwa; AEH Desouky. *J. Nanomaterials*, **2014**, Article ID 431089
[9] R De Philippis ; M Vincenzini. *FEMS Microbiol. Rev*., **1998**, 22, 151-175.
[10] R De Philippis; C Sili; R Paperi; M Vincenzini. *J. Appl. Phycol*., **2001**, 13, 293-299.
[11] S Ozturk; B Aslim; Z Suludere. *Bioresour. Technol*., **2009**, 100, 5588-5593.
[12] J Bender; P Phillips. *Bioresour. Technol*., **2004**, 94, 229-238.

[13] CS Freire-Nordi; AAH Vieira; OR Nascimento. *Process Biochem*., **2005**, 40, 2215-2224.
[14] S Ozturk; B Aslim. *Environ*. *Sci*. *Pollut*. *Res*. *Int*., **2008**, 15, 478-480.
[15] RY Stanier; R Kunisaw; M Mandel; G Cohen-Baziere. *Bacteriol*. *Rev*., **1971**, 35, 171-205.
[16] JL Plude; DL Parker; OJ Schommer; RJ Timmerman; SA Hagstrom; JM Joers; R Hnasko. *Appl*. *Environ*. *Microbiol*., **1991**. 57, 1696-1700.
[17] JH Klock; A Weiland; R Seifert; W Michaeli. *Mar*. *Biol*., **2007**. 152, 1077-1085.
[18] RG Spir. Analysis of sugars found in glycoproteins. *Method Enzymol*., **1966**, 8, 3-26.
[19] TD Herbert; PJ Phipps; RE Strange. *Anal*. *Biochem*., **1971**, 19, 119-132.
[21] Y Bar-or; M Shilo. *App.l Environ*. *Microbiol*., **1987**, 53, 2226-2230.
[22] SO Avijeet; G Oinam; KO Singh; ON Tiwari. *Int*. *J*. *Res*. *BioSci*., **2013**, 2(2), 75-82.
[23] U Nubel; F Garcia-Pichel; G Muyzer. *Appl*. *Environ*. *Microbiol*., **1997**, 63(8), 3327-3332.
[24] JD Thompson; DG Higgins; TJ Gibson. *Nucleic Acids Res*. **1994**, 22, 4673-4680.
[25] RV Eck; MO Dayhoff. National Biomedical Research Foundation, Silver Springs, Maryland. **1966.**
[26] M Nei; S Kumar. Oxford University Press, New York. **2000.**
[27] K Tamura; J Dudley; M Nei; S Kumar; *Mol*. *Biol*.*Evol*., **2007**, 24, 1596 1599.
[28] RD Monds; PD Newell; RH Gross. *Mol*. *Microbiol*., **2007**, 63, 656-679.
[29] JS Khattar; DP Singh; N Jindal; N Kaur; Y Singh; P Rahi; A Gulati. *App*. *Biochem*. *Biotechnol*., **2010**, 162, 1327-1338.
[30] B Nicolaus; A Panico; L Lama; I Romano; MC Manca; A De Giulio; A Gambacorta. *Phytochemistry*, **1999**, 52, 63-647.
[31] S Singh; S Das. *World J*. *Microbiol*. *Biotechnol*., **2011**, 27, 1971-1980.
[32] R Mota; R Guimaraes; Z Buttel; F Rossi; G Colica; CJ, Silva; C Santos; L Gales; A Zille; R De Philippis; SB Pereira; P Tamaqnini. *Carbohyd*. *Polym*., **2013**, 92, 408-1415.
[33] A Otero; M Vincenzini. *J*. *Biotechnol*., **2003,** 102, 143-152.
[34] WK Kroen; WR Rayburn. *J*. *Phycol*., **1984**, 20, 253-257.
[35] J Moreno; MA Vargas; JM Madiedo; J Munoz. *Biotechnol*. *Bioeng*., **2000**, 67, 283-290.
[36] ON Tiwari; KH Romi; SH Minerva; SS Aribam; O Gunapati; JB Jerry. *App*. *Biochem*. *Biotechnol*., **2015**, 176(7), 1950-1963.
[37] TP West. *J*. *Basic Microb*., **2011**, 51, 673-677.
[38] N Jindal; DP Singh; JS Khattar. *J*. *Basic Microbiol*., **2013**, 53, 902–912.
[39] IW Sutherland. *Biotechnol*. *Adv*., **1994,** 12, 393-448.
[40] P Raus Madieldo; CG De Los Reyes Gavilan. *J*. *Dairy Sci*., **2005**, 88, 843-856.
[41] R De Philippis; G Colica; E Micheletti. *Appl*. *Microbiol*. *Biotechnol*., **2011**, 92, 697-708.
[42] CG Kumar; H Joo; R Kavali; J Choi. *World J*. *Microbiol*. *Biotechnol*., **2004**, 20, 833-836.
[43] A Herrero; A Muro-Pastor; E Flores. *J*. *Bacteriol*., **2001**, 183, 411-425.