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Extraction and Partial Characterization of Exopolysaccharides from Marine Cyanobacteria and their Flocculation Property

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ABSTRACT

The present study focused on the extraction of cyanobacterial exopolysaccharides and their utilization as a bioflocculant. A cyanobacterium, *Phormidium* sp., was chosen based on the production of Extracellular Polymeric Substances (EPS). Later it was identified as *Phormidium persicinum* by 16S rDNA gene sequence and sequences were deposited in GenBank with accession number KC 859032. EPS was extracted from this strain using standard precipitation method. It was found that EPS production was maximum at late log phase of cyanobacterial growth (20 days). HPLC analysis revealed that presence of sucrose as a major component in the extracted EPS. Interestingly, the extracted EPS was found to be a good bioflocculant even at very low concentration (10 mg L^{-1}). Cyanobacterial based EPS showed potential bioflocculation which can be directly utilized for the water purification and refining processes.

Key words: Cyanobacteria, *Phormidium* sp., EPS, FTIR, HPLC, flocculation

INTRODUCTION

Over the last two decades there has been an expanding interest in polysaccharides produced in extracellular by microorganisms for food, pharmaceutical and medical use, including vaccines. Cyanobacteria also known as blue green algae are a group of extraordinarily diverse Gram-negative prokaryotes that originated 3.5 billion years ago. Their diversity ranges from unicellular to multicellular, coccoid to branched filaments, nearly colorless to intensely pigmented, autotrophic to heterotropic, psychrophilic to thermophilic, acidophilic to alkylophilic, planktonic to barophilic, freshwater to marine including hypersaline (Thajuddin and Subramanian, 2005). Owing to their ecological and biochemical diversity, cyanobacteria, as well as several species of microalgae have been regarded as good candidates for various biotechnological applications such as antibacterial compounds, nanoparticles synthesis and dye decolorization (Mubarak Ali *et al*., 2008; MubarakAli and Thajuddin, 2009; MubarakAli *et al*., 2011, 2012). EPS play a crucial role in biosorption and binding of heavy metals, effective absorbent for removing organic pollutants such as dyes and pesticides (Bhatnagar *et al*., 2012). Exopolysaccharides are high-molecular-weight of polymers secreted by a microorganism into the surrounding environment that are composed of

sugar residues. Cyanobacteria can be included among the potential sources of new polymers; several species have been characterized by the presence of thick capsules surrounding the cells and by the ability to release polysaccharide material into culture medium (Khattar *et al.*, 2010; Bhatnagar *et al.*, 2012). It was reported earlier that a large number of cyanobacteria are characterized by the presence of polysaccharidic outermost investments (Wingender *et al.*, 1999; Wolfaardt *et al.*, 1999).

It has been reported that the synthesis of exocellular polysaccharides in microorganisms, including cyanobacteria plays a major role in protecting cells from stress in extreme habitats from other harmful conditions. Many cyanobacteria were capable to overcome the stress from desiccation or due to low water activity in desert or other hypersaline areas (Tamura *et al.*, 2011).

In recent years, attention towards cyanobacterial EPS has increased because a large number of cyanobacteria are characterized by the presence of polysaccharide outer investments and these organisms are photosynthetic easy to culture, some are even N_2 fixers and are able to manipulation of conditions for enhancing growth and/or EPS production. Cyanobacterial polysaccharides are composed of monosaccharides and are characterized by the presence of pentoses (usually absent in polysaccharides of prokaryotic origin), acidic sugars (glucuronic and/or galacturonic acids) anionic organic (acetyl, pyruvil) and inorganic (phosphate and sulphate) substituents (Challouf *et al.*, 2011). It has been suggested that EPS produced by cyanobacteria could be useful in various applications such as water holding capacity of soil and removal of heavy metals and solid materials from water reservoirs (Ozturk and Aslim, 2008). The present study focused on the extraction and partial characterization of extracellular polysaccharides from a marine cyanobacterium, *Phormidium persicinum* and an insight into its potential as bioflocculant.

MATERIALS AND METHODS

Microorganism and culture conditions: Sample was obtained from the Germplasm of Department of Microbiology Bharathidasan University, India. The culture was maintained in MN+medium (Rippka *et al.*, 1979), under white fluorescence lamps 45 μmol $m^2 sec^{-1}$ at 25±2°C.

DNA extraction and 16S rRNA gene amplification: The extraction of genomic DNA from cyanobacterial isolates were carried out (Smoker and Barnum, 1988). PCR amplification was performed for the purified DNA using CYA 106 (5'-CGG ACG GGT GAG TAA CGC GTGT-3') as forward and CYA 781 (5'-GAC TAC TGG GGT ATC TAA TCC CA T-3') as reverse primers. The polymerase chain reaction conditions include initial denaturation of template DNA at 94°C for 2 min. Further denaturation was carried out at 94°C for 5 sec; annealing at 47°C for 10 sec, elongation at 72°C for 30 sec for 40 cycles and final elongation at 70°C for 7 min. Amplified products were isolated by electrophoresis on 1.2% agarose gel using 1X TAE buffer at a constant supply of 100 V for 30 min. Sequencing was done with amplified samples with the respective forward and reverse primers and sequences were submitted to GenBank via BankIt submission tool (Pandiaraj *et al.*, 2012).

Determination of growth: The pigment content such as Chlorophyll a was estimated by extraction with acetone using Lichenthaler equation (Lichtenthaler, 1987).

Extraction of exopolysaccharide: Cyanobacterial culture was drawn from the growth media in late log phase. Biomass was harvested by using Whatman No. 1 filter paper and the filtrate containing media and other exopolymeric substances. The filtrate was centrifuged at 8000 g for 20 min at 4°C. Equal volume of acetone was added to the cell free filtrate and kept at 4°C for

48 h. After incubation, it was then centrifuged at 8000 g for 20 min at 4°C (Vicente-Garcia *et al.*, 2004). The obtained substance was then lyophilized and resulted dried substances contains EPS was subjected to further analytical procedures.

FTIR analysis: The molecular structure of polysaccharide was identified partially by using the FTIR analysis. The important functional groups in the EPS were detected using Fourier transform infrared spectroscopy. The sample was prepared by grinding dry Kbr with EPS in the ratio of 10: 0.1 w/w and pressing it in a mold. The FT-IR spectra were recorded in transmittance mode on a Perkin Elmer Spectrum Two system at the range of 400 to 4000 cm^{-1} (Perkin Elmer, USA).

HPLC analysis: The separation and identification of the sugar compounds present in the EPS were done by HPLC analysis. About 20 mg of each lyophilized form of EPS sample were mixed with 2 mL of 0.5 M trifluoroacetic acid and heated for 4 h at 100°C in hot water bath. After cooling the mixture was extracted with diethyl ether. The organic phases were discarded and the aqueous phase was then evaporated to dryness by rotary evaporation at 60°C. This extract was dissolved in 1 mL of distilled water and was analyzed for HPLC. The chromatographic conditions were as follows: Liquid chromatogram with Uv-Vis detector, Column CD18 and the mobile phase was acetonitrile-H_2O (80:20), Flow rate was 0.7 mL min^{-1}.

Flocculation property: The flocculation property of cyanobacterial EPS was explored by analyzing its ability to sediment the fine clay slurry. Briefly, clay was dissolved in distilled water in a final concentration of 50 g L^{-1}. Equal volume of finely dissolved clay solution was transferred into two separate beakers. One beaker is kept as control and another one is taken as the test. The 100 mg of EPS was added in test solution and both the beakers were vortexed thoroughly for 5 min. After homogenization both the beakers were kept standby for settling. The Optical Density (OD) of the upper clear layer (A) was measured with a spectrophotometer at 550 nm. An experiment with distilled water instead of EPS was used as control (B). The flocculating property was calculated by using the following equation (Deng *et al.*, 2003):

$$\text{Flocculating property (\%)} = \frac{B - A}{B} \times 100$$

Where:
A = Optical density of the sample at 550 nm
B = Optical density of control at 550 nm

The effect of varying the concentration of EPS on flocculating property was also monitored. Different concentration of EPS was made by adding 10, 20, 30, 40 and 50 mg in 1 mL of distilled water in separate test tubes. The 5 mL of clay slurry having a concentration of 0.04 g mL^{-1} of distilled water was added in each test tubes and vortexed thoroughly. After homogenization it was allowed to stand for a time period of 10 min and observe the flocculation ability.

RESULTS AND DISCUSSION

Morphological characterization: An unidentified marine cyanobacteria was morphologically identified as *Phormidium persicinum* based on the morphological characteristics using standard cyanobacterial monographs (Fig. 1). The morphological characters were determined with the cell

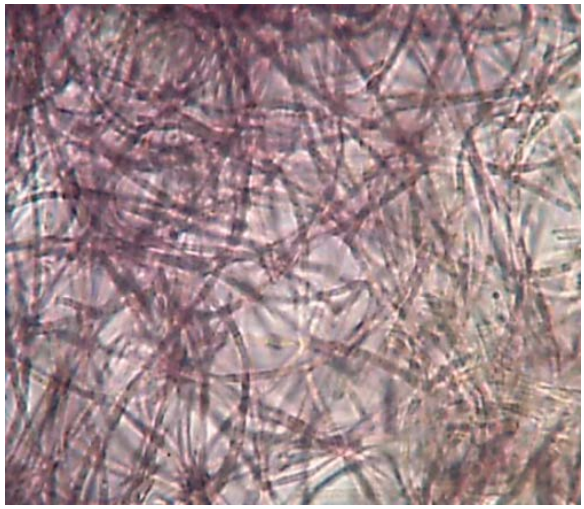

Fig. 1: Microphotograph of *Phormidium persicinum*

shape and size comparison with standard monograph. The culture was cultured in mass quantity and was maintained in the Germplasm as stock culture for further experiments.

Molecular characterization

16S rDNA gene amplification and sequencing: *Phormidium persicinum* showed significant clear bands in electrophoresis which were used for the amplification of 16S rDNA gene in PCR. The gel picture clearly depicts the amplified region is around 600 bp in length (Fig. 2). The amplified samples were then sequenced. The sequences results revealed that 600 kb of 16S rDNA gene coding sequences were observed and analyzed. The 16S rDNA gene sequence was aligned and the sequence similarity with existing database was determined using BLAST. The sequence was deposited in GenBank (NCBI) with accession number KC859032.

Phylogenetic analysis: Phylogenetic analysis helps in understanding the evolutionary relationship between microorganisms. Phylogenetic tree helps to study the evolutionary relationship of and between different groups of species. Based on the sequence data a phylogenetic tree was created by using bioinformatics methods like neighbor joining and UPMGA. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length is 0.89130435 (Fig. 3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 46 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

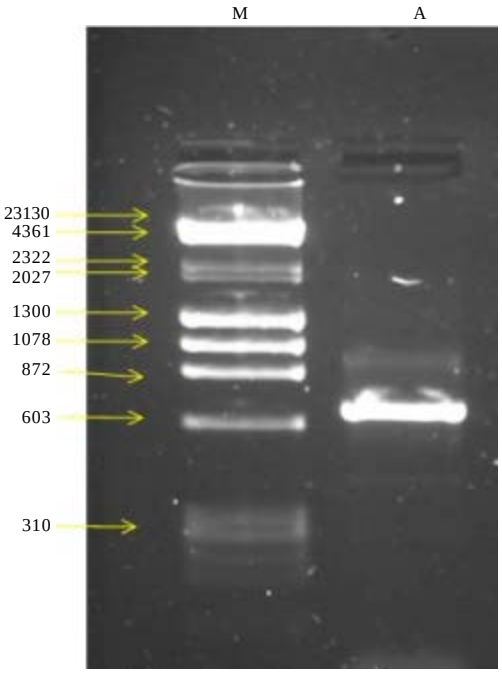

Fig. 2: 16S r DNA gene amplification of *Phormidium persicinum*. M: Marker, A: amplified product

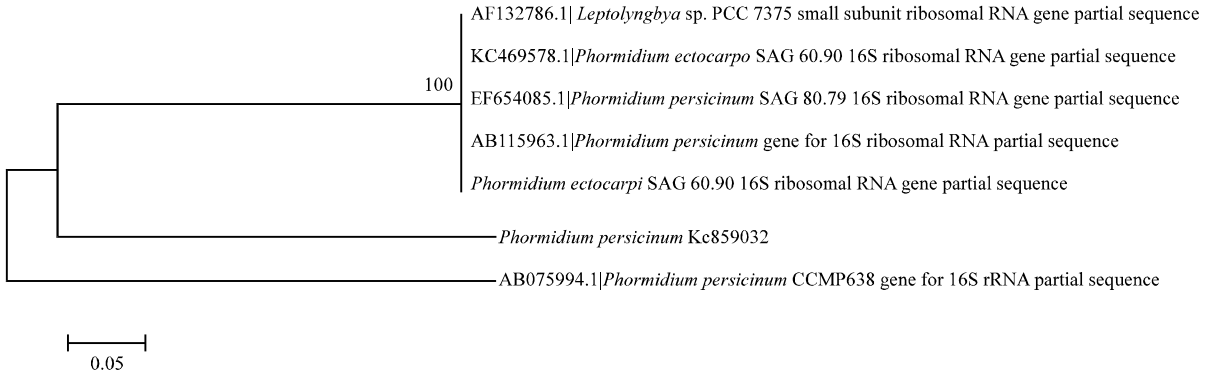

Fig. 3: Evolutionary relationship of *Phormidium persicinum* (KC859032)

Restriction site analysis: The restriction site analysis was done for the 16S rDNA gene sequence of *Phormidium persicinum*. It was found that 53% of GC content and 47% of AT content. Addition to that it has more than 40 restriction sites. In which there was no repetition of restriction enzyme cleaving sites were found (Fig. 4).

Growth rate: Chlorophyll was estimated to determine the growth rate of the cultures in an interval of 4 days. The culture has shown a significant growth for a period of 20 days. After incubation, it was found that the cultures entered its decline phase. The growth curve was shown (Fig. 5).

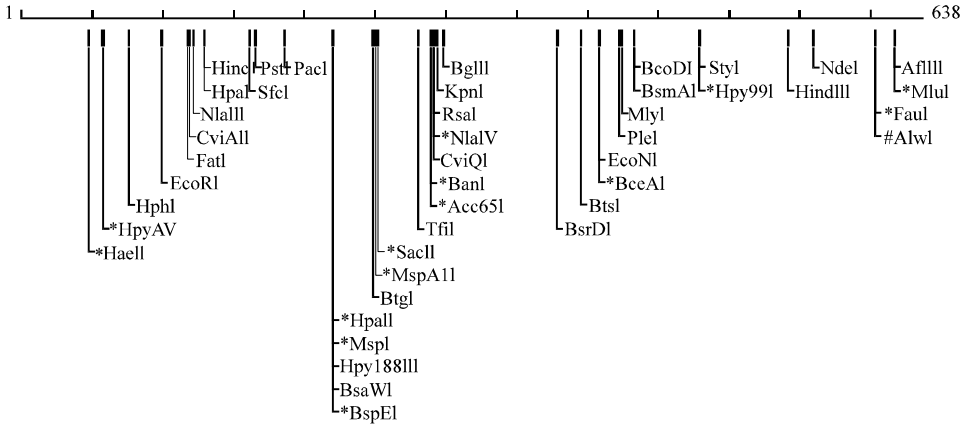

Fig. 4: Restriction site analysis of *Phormidium persicinum*. (KC859032)

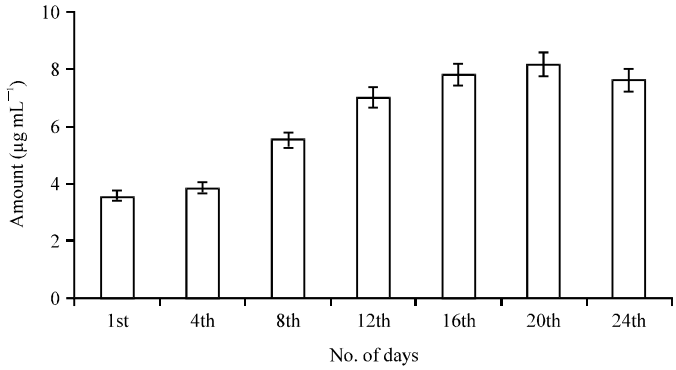

Fig. 5: Estimation of Chlorophyll a content in *Phormidium persicinum*

Extraction and characterization of EPS: Exopolysaccharides were extracted from the strain using the acetone precipitation method. Extraction was done at the end of log phase of each culture. The obtained EPS was further analyzed by different analytical techniques. The basic nature of EPS is that they are insoluble in solvents, thus 20 mg of the sample was taken in a tube and were tested for solubility in solvents like methanol, ethanol, acetone without any treatment. The EPS were found not to dissolve in these solvents and settled down at the bottom of the tube. These tests hence gave more confirmation to the nature of polysaccharides.

Fourier Transform Infrared Spectroscopy (FTIR) analysis: The IR spectra for the EPS were obtained to determine the surface functional groups by using FTIR spectrophotometer (Perkin Elmer, USA) (Baldev *et al*., 2014). The presence of different kinds of functional groups was revealed in the EPS extracted (Fig. 6). According to the literature (Vicente-Garcia *et al*., 2004) the IR spectrum of the EPS produced by *Phormidium* 94a exhibited a peak around 3430 cm^{-1} is assigned to OH stretching frequency and the absorption at 2928 cm^{-1} is attributed to C-H stretching frequency. Absorption at 1600 cm^{-1} is assigned to the stretching vibration of the carboxylate group. The absorption at 1408 cm^{-1} is possibly due to symmetric CH_3 bending. Interestingly, *Phormidium persicinum* shown peaks at 3833, 3417 cm^{-1} correspond to alcoholic

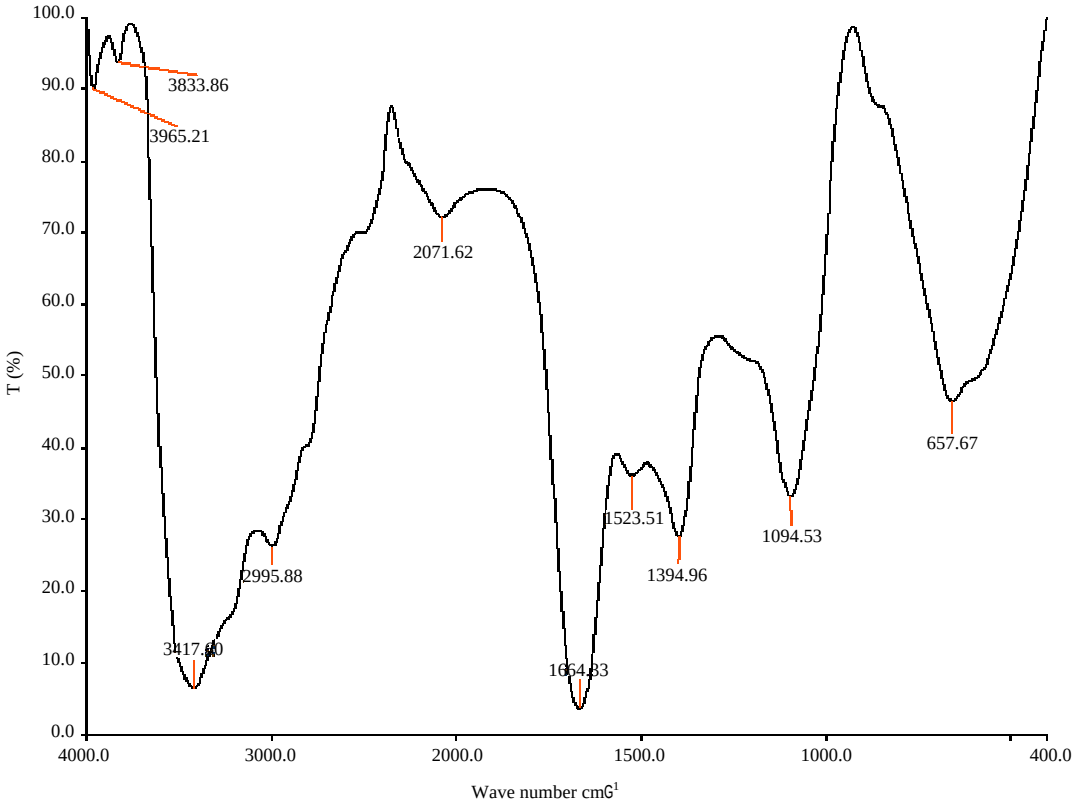

Fig. 6: FTIR analysis for extracted EPS from *Phormidium persicinum*

Table 1: Functional group assignment to the extracted EPS using IR spectrum

Wave No. (cm^{-1})	Assignment	Functional group
3833	O-H stretch	Alcohols, phenols
3417	O-H stretch, H-bonded	Alcohols, phenols
2999	C-H stretch	Alkanes
2071	-C (triple bond) C-stretch	Alkynes
1664	C = O stretch	Carbonyls (general)
1523	C-C stretch (in-ring)	Aromatics
1394	C-H rock	Alkanes
1094	C-N stretch	Aliphatic amines
657	C-Br stretch	Alkyl halides

group vibrations while peak 2999 cm^{-1} corresponds to alkane group vibrations while peaks 2071 cm^{-1} corresponds to alkynes group vibrations, peaks like 1664 cm^{-1} corresponding to carbonyl (general) group vibrations while peaks of 1523 and 1523 cm^{-1} corresponds to the aromatic and alkane group vibrations (Table 1). Here also the *Phormidium* sp. has been used and its shows some of its vibrations exactly matching with earlier reports by Vicente-Garcia *et al.* (2004) and Khattar *et al.* (2010) and so on. In contrast some new peaks indicating the difference in the molecular structure also have been reported in this study.

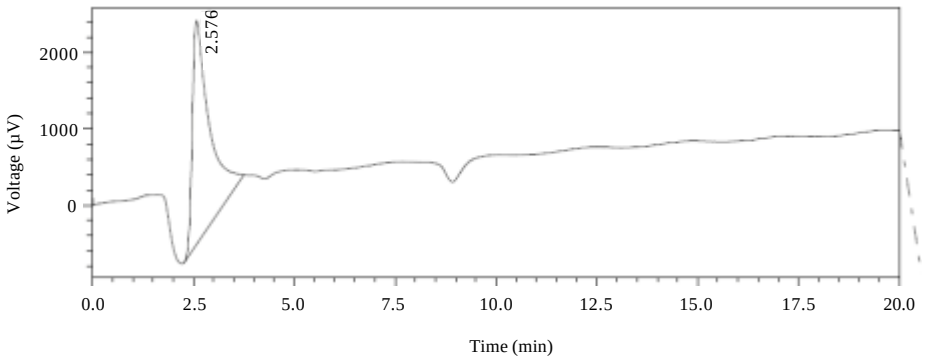

Fig. 7: HPLC analysis for extracted EPS from *Phormidium persicinum*

High performance liquid chromatography analysis: The extracted EPS was analyzed by HPLC to identify the compound groups present in it. The HPLC analysis shown in that the peak values of the sample were correlated with the standard sample peak values. The peak values of samples are tabulated and are compared with standard Rf values. HPLC analysis of hydrolyzed samples of EPS has shown the presence of neutral sugars glucose/mannose, ribose and rhamnose. The identification of three sugar components by HPLC and the fourth sugar was identified by TLC technique and the study confirms that presence of sugar components in EPS extracted from cyanobacteria (Khattar *et al.*, 2010). The Rf values of sample was compared with standard sample Rf values which are 2.577 and 2.508, respectively (Fig. 7). Extracted EPS obtained from the samples was identified as glucose residues.

Flocculation property: The production of extracellular bioflocculant by benthic cyanobacteria is of considerable ecological importance. It was revealed that EPS amended suspension was cleared off rapidly when compared to the control. It was found that the test sample with the amended EPS was cleared by suspend the dissolved particles in a very short period of time. At the same time the control (without the EPS) was found to be dispersed as such for a long period of time. Same experiment was carried out with various concentrations of EPS, it was found that as the concentration of EPS increases the flocculating activity also increased (Fig. 8).

Flocculation and aggregation of suspended particles by bioflocculant might have a critical role in allowing light penetrate to the sediment water interface, thus facilitating the survival and growth of the benthic cyanobacteria that occupy a low-light zone (Fattom and Shilo, 1984; Bender *et al.*, 1994). It was evidenced that flocculation process induced by bioflocculants was based on the bridging mechanism in which some positive charged ions may decrease the negative electrical charge density of the clay particles and of the bioflocculant molecules and thus enhances the flocculation process. A bioflocculant produced by *Sphingomonas paucimobilis* was removed dye very effectively and industrial effluents containing dyes could be treated before discharge into the environment (Wong *et al.*, 2003). The flocculating property of EPS can be utilized to solve many environmental problems such as water pollution, drainage problem and industrial effluent discharges etc. Flocculating activity of EPS can be suggested as a green chemistry procedure for the purification of waste water and thus making it available for our routine usage.

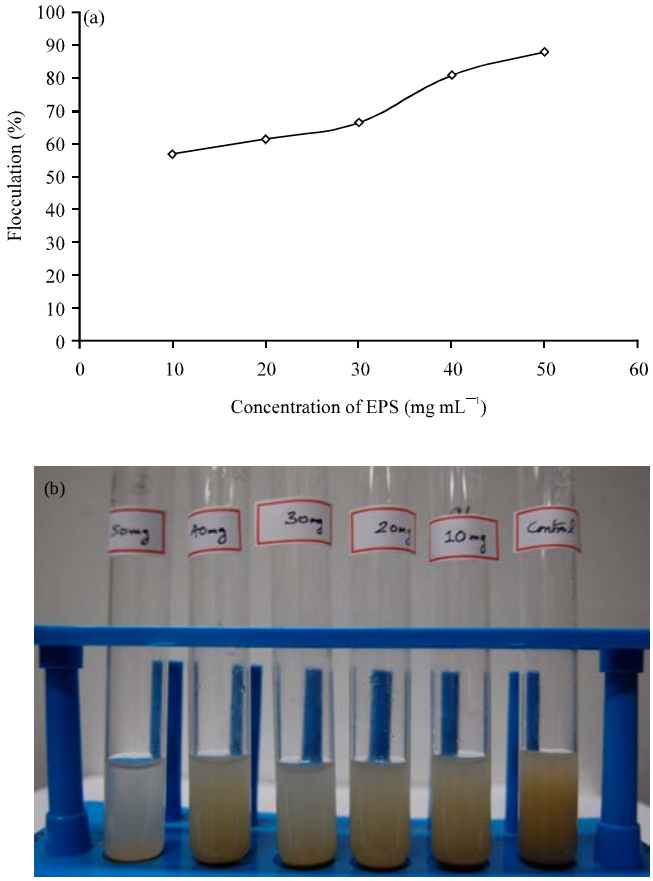

Fig. 8(a-b): Experimental set up (a) Increasing flocculating activity with increasing concentration of EPS (b) Increasing flocculating activity from 10-50 mg mL^{-1} of EPS

CONCLUSION

There has been an expanding interest in polysaccharides produced by cyanobacteria for food, pharmaceutical and medical use. EPS producing cyanobacteria was chosen from the collection. It was morphologically and molecularly identified as *Phormidium persicinum*. Extraction of EPS was done from the growth media of *P. persicinum*. The obtained EPS was characterized and found that aldehyde, hydroxyl groups, sugar residues by FTIR and HPLC analysis, respectively. Additionally, extracted EPS possesses potential flocculation property. It was determined that EPS at concentration (10 mg L^{-1}) showed ideal bioflocculant. Cyanobacterial based EPS provides a new ecofriendly approach for the purification of water resources.

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