PROFORMA FOR SUBMISSION OF UGC-MRP FINAL REPORT

Title of the project

Molecular characterization and the effect of backwater cyanobacterial exopolysaccharides on saline soil reclamation

UGC Reference No & Date

42-483/2013 (SR) dated, 22nd March 2013

Area of Specialization: Microbiology

Submitted to

ज्ञान - विज्ञानं विमुक्तये

UNIVERSITY GRANTS COMMISSION
BAHADURSHAH ZAFAR MARG
NEW DELHI – 110 002

Submitted by

Dr. G.SELVAKUMAR
ASSISTANT PROFESSOR IN MICROBIOLOGY,
DDE
ALAGAPPA UNIVERSITY
KARAIKUDI - 630 004
TAMILNADU – INDIA

Enclosure 1

Introduction

Soil salinity is a major problem associated with soil management in arid and semi-arid regions worldwide and is potential threat to the existing agricultural technology in all continents (Szabolcs, 1979). About 410 million hectares of the earth are reported to be saline and alkaline nature. In order to reduce the salinity of the soils without affecting its physical nature, biological method is very suitable, easiest and cost effective.

Cyanobacteria is the largest, the most diverse and the most widely distributed group of photosynthetic prokaryotes and they cope up with wide spectrum of global environmental stress such as heat, cold, desiccation, salinity, nitrogen starvation and osmotic stress (Sinha and Hader, 1996). Particularly, backwater cyanobacteria have the ability to tolerate and adapt in all seasons with wide range of salinity (Selvakumar and Sundararaman, 2009). The Principal Investigator has used the predominant species such *as Phormidium fragile* and *Oscillatoria curviceps* which were present throughout the year for reclamation of salt affected soil. Reclamation studies showed that the pH. Electrical conductivity (EC) and Exchangeable Sodium percentage (ESP) were significantly reduced in saline-alkali soil (Selvakumar and Sundararaman, 2009).

Cyanobacteria have been recognised as an important agent in the stabilisation of soil surfaces primarily through the production of extracellular polysaccharides, which are prominent agents in the process of aggregate formation and increase in soil fertility (Hu et al. 2003; Acea *et al.,* 2003; Pandey *et al.,* 2005). Some N-fixing cyanophyceae that produce extracellular polysaccharides and this division offers potential for the development of soil conditioners and increased productivity (Malam et al. 2007). The present study will be planning to apply cyanobacterial exopolysaccharides in alkaline soil and to determine the role of cyanobacterial exopolysaccharides in soil reclamation and crop yield.

Methodology used in this study

Organism and Culture Conditions

Cyanobacterial strains PBC1, PBC2, PBC3, PBC4, PBC5, PBC6, PBC7, PBC8, PBC9, PBC 10 and PBC11 were isolated from Kattumavadi Backwater of Palk Strait region, TN, India and maintained in 100ml Erlenmeyer flask containing 50ml of Artificial Sea Nutrient III liquid medium (ASN III), containing the salts (gmL-1), NaCl 25gm; MgCl2, 6H2O 2 gm; KCl 0.5 gm; NaNO3 0.75 gm; K2HPO4,3H2O 0.02 gm; MgSO4,7H2O 3.5 gm; CaCl2 0.5 gm; Citric Acid 0.003 gm; Ferric Ammonium Citrate 0.003 gm; EDTA 0.0005 gm; Na2CO3 0.02 gm; Trace Metal Mix A5 1 ml containing (mg mL-1) H3BO3, 2.86 mg; MnCl2,4H20, 1.81 mg; Na2MoO4;2H2O, 0.390 mg; ZnSO4;7H2O, 0.222 mg; CuSO4;5H2O, 0.079 mg and Co(NO3)2;6H2O, 0.0494 mg. The pH was maintained at 7.5 after sterilization. The culture sets are maintained by regular transfers into fresh liquid medium at 20˚C in 16/8 hour dark/light cycle under cool fluorescent light having light intensity 20-30 µmol photons m^{-2} s (Rippka *et al.,* 1979). The cultures were made free of diatoms and green algae by adding 0.16mM of cyclohexamide final concentration for 24 h with incubation in light. A combination of ampicillin-streptomycin was also used at a final concentration of 40µg/ml and 100µg/ml respectively to make it free from bacterial flora.

Standardization of EPS Extraction

Most of the extraction procedure so far reported, dealt with EPS which were released in the culture medium. The main obstacle faced here is that the filamentous algal strain, do not release EPS in this way. The extraction procedure was modified from the standard protocols (Joana and Rosario, 1996) for better extraction. Standardization of polysaccharide extraction the following method was used. For RPS (Released Polysaccharide) extraction, the cell free supernatant was treated with two different solvent (ethanol and isopropanol) and 3% CTAB and kept it for 4^0C. The precipitated polysaccharide was collected by centrifugation at 15000x g for 20 min. For CPS (Cell bound Polysaccharide) extraction, cyanobacterial biomass were treated with 0.6M NaCL + 0.06M EDTA, 4M NaOH and hot water and incubate it for 80^0C in water bath for 1hour. The cell-free supernatant was collected by centrifugation and to it, two different solvent (ethanol and isopropanol) and 3% CTAB and kept it for 4^0C for polysaccharide precipitation. The precipitated polysaccharide was collected by centrifugation at 15000x g for 20 min. Precipitated polysaccharides were quantified by the Anthrone method (Morris, 1948).

Mass cultivation

Out of 11 Strains, EPS producing four strains were mass cultivated and analyzed in regular interval. Growth OD (Optical density at 670nm), RPS production and CPS production were measured using standard protocols.

Extraction and Purification of EPS

Cell free supernatant of both RPS and CPS was kept for protein precipitation with the addition of 10% TCA (Tri Chloro Acetic Acid) at -20^{0}C for 24h. Both RPS and CPS supernatant was extracted and evaporated to half volume and dialyzing (membrane with 3,500 Da cutoff) against distilled water for 72 h. To the dialyzed solutions, equal volumes of chilled solvent (ethanol or isopropanol) were added, mixed, and kept in a deep freezer (−20°C) for 72 h. Precipitated EPS was washed with chilled ethanol and centrifuge at 15000 rpm for 15 min and the pellets were lyophilized for further analysis (Khattar *et al.*, 2010).

Carbohydrate Estimation

For quantitative determination RPS and CPS, a phenol-sulfuric method by Dubois, *et al.,* (1956) was applied as follows: For standard, different glucose standard solutions (0.30, 0.25, 0.20, 0.15 and 0.10)mg/mL were prepared from glucose stock solution of 1.00mg/mL. A volume of 0.40 mL of each solution (standard solutions and the extracted polysaccharides) was transferred into glass test tubes separately, then 0.40 mL of 5%phenol solution and 2.00 mL concentrated sulfuric acid were added to all tubes, mixed well then shook for 30 minutes and finally the absorption for all tubes and the blank (distilled water with the reagents) were measured at 490 nm wavelength. A standard curve was plotted with concentrations verses absorption.

Characterization of exopolysaccharide

Light Microscopy

Cells were washed with distilled water, placed in 3% acetic acid for 30 min, and then placed in Alcian blue reagent (pH 2.5) containing 0.33% (wt/wt) Alcianblau 8GS (Chroma) in 3% (wt/wt) acetic acid to stain carboxylic polysaccharides. The samples were washed with distilled water to remove excess dye, and the stained samples were observed by light microscopy (Nikon).

Scanning Electron Microscopy

For SEM analysis the cyanobacterial glass slides were washed with PBS and fixed by 2.5% glutaraldehyde (30 mins). After fixations the samples were washed with PBS and serially dehydrated with different concentrations of ethanol (20%, 40%, 60%, 80%, and 100%) for 10 min each. Followed by dehydration the samples were gold sputtered and analyzed with a scanning electron microscope (Quanta FEG 250, FEI, Netherlands).

Confocal laser scanning microscopic (CLSM)

The glass slides of four strains were analyzed under CLSM, slides were washed with 0.1 M PBS and stained with acridine orange (0.1%) and the cyanobacterial EPS were visualized using confocal laser scanning microscope (CLSM) (Zeiss LSM 710, Carl Zeiss, Germany). Zeiss LSM Image Examiner (Version 4.2.0.121) was used for processing the cyanobacterial slides which had an excitation filter 515–560 and magnification at 20 × and the Z stacks analysis (surface topography and three-dimensional architecture) was done with the Zen 2009 software (Carl Zeiss, Germany).

Fourier Transform Infrared spectroscopy.

For Fourier transform – infrared spectroscopy (FT-IR), pellets were obtained by grinding a mixture of 2 mg EPS with 200 mg dry KBr and pressing the mixture into a 16-mm-diameter mold. FT-IR spectra were recorded on a Thermo Scientific Nicolet is 5 instrument with a resolution of 4 cm^{-1}. Spectra were obtained in the 4000-400 cm region.

Optimization of photoperiods and pH for polysaccharide production

In order to evaluate the influence of pH, Adjust the pH of production medium 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 using 1.0 N HCL and 1.0 N NaOH, keeping the final volume of the medium to 100 ml. Adjustment of pH will be done before sterilization. For evaluating the effect of different photoperiods, light periods ranging from 4 to 24 h with a photon flux density of 35µEm-2s-1 will be experimented upon. Treat the cultures with different light and dark cycle i. e. Continuous light (24 h), 16 h light 8 h dark period, 12 h light 12 h dark period, 8 h light 16 h dark period, 4 h light 20 h dark period.

Seed germination experiment with and without NaCl

The seeds were taken in 44 Petri dishes (Positive control- 16, Negative control- 4, Treated- 24) and subjected to surface sterilization with 2% $HgCl_2$ .Twenty five seeds per Petri plate were placed at different salt concentrations (0.1M, 1M and 2M). One set of the Petri dishes were amended with 5 ml of concentrate Cyanobacterial EPS while the other set without amendment served as control. Response of different crops under various salinity levels were assessed as % seed germination, and total leaf chlorophyll in 7 days old seedlings. The chlorophyll content was estimated by hot extraction with methanol following Mckinney (1941).

Seed germination (%) = (no. of germinated seeds / no. of seeds in Petri-dish) x 100

Estimation of Chlorophyll

The chlorophyll content was estimated according to the method of Amon (1949). 1g of fresh leaves were homogenated in 5ml of 80 percentage acetone and 1ml of 0.5 percentage $CaCO_3$ solution using a mortar and pestle. The extract was filtered using a cotton cloth. The debris was washed with fresh acetone until it became colorless. Then the extract was centrifuged at 5000 rpm for 20min. the supernatant was made up to a known volume. It was used for the estimation of chlorophyll by measuring the optical density at 643 and 663 nm using spectrophotometer.

Preparation of the algal filtrate

The cyanobacteria were rinsed with fresh water for 5–10 min to remove sand particles, debris, macroscopic epiphytes, shells and other impurities. They were shade dried for a week and prepared as coarse powder with a mixer grinder. Powdered culture (0.5 kg) was suspended in sterile double distilled water (1:2 w/v), stirred for 60 min and autoclaved at 121 °C for 30 min. Autoclaved extract was filtered through muslin cloth, allowed to cool at room temperature and then filtered through Whatman no. 1 filter paper and used for further experimental studies.

Seed germination experiment for algal filtrate with and without NaCl

Seeds were surface sterilized with 0.1 % $HgCl_2$ for 3 min. Ten viable seeds were tested for each algal aqueous extract. Seeds, without algal extract, served as control. Each petri dish contain ten surface sterilized seeds were placed on filter paper and moistened with 10 ml of the aqueous extract of algae with different salt concentrations (0.1M, 1M and 2M).

Petri dishes containing seeds with 10 ml of distilled water served as a control. The growth parameters including germination percentage, root length and shoot length were recorded on the 3 days after incubating seed at 280C (Pitchai et al. 2010).

Molecular Characterization

Selected cyanobacterial genomic DNA was isolated by using the InstaGeneTM Matrix Genomic DNA isolation kit according to the manufacturer's instructions. 16S rRNA genes were amplified (MJ Research PTC-225 Peltier Thermal Cycler) using specific forward and reverse primers (Padmini et al., 2020). Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore). The purified PCR products (about 1500 bp) were sequenced using Big-Dye Terminator v.3.1 Cycle Sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA). The 16s rRNA sequences were blasted using NCBI blast similarity search tool ((http://www/ncbi.nlm.nih.gov/). The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Results and discussion

Cyanobacterial samples were isolated from Backwater of Palk Strait region (figure 1), Tamil Nadu, India. Cyanobacterial samples were isolated from the Thondi coast which included (figure 2) Mimisal bridge (a), Kattumavadi (b), Kodiyakarai (c) and Cheethapattinam (d).

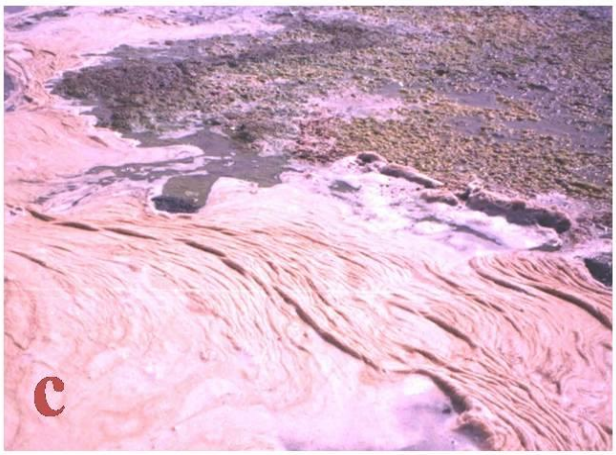

Figure 1: **Study sites of backwater of Palk Straight Region (Thondi)**

Figure 2: Samples collected from thondi coast - (a) Mimisal bridge, (b) Kattumavadi, (c) Kodiyakarai and (d) Cheethapattinam.

The cyanobacterial diversity was assessed in four back water area of Palk Straight Region (figure 2). Totally, 11 cyanobacterial stains (figure 3) were isolated during study period. They are named as, PBC5, PBC6, PBC7, PBC8, PBC9, PBC 10 and PBC11. All the cultures showing the ability to produce EPS was released into the medium and it could be separated easily after precipitation. Apart from 11 strains, four strains (PBC3, 7, 8 & 9) were selected for further studies based on their adaptability to the laboratory condition and fast growth compared to the other strains. These strains were morphologically identified as PBC 1, PBC 2, PBC and PBC 4 through light microscopy.

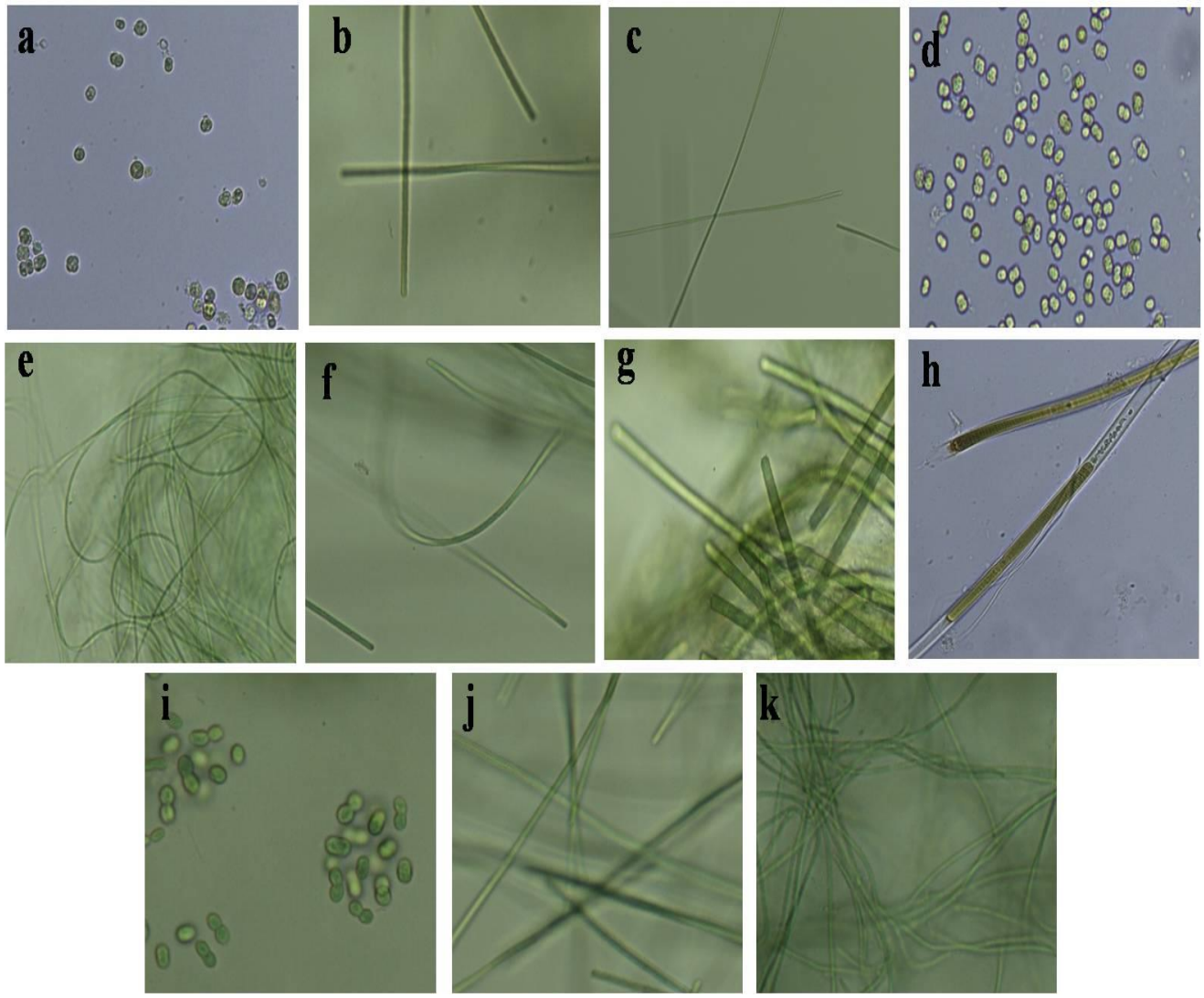

Figure 3: Light microscopic picture of isolated cyanobacterial strains (PBC1 - a, PBC2 - b, PBC3 - c, PBC4 - d, PBC5 - e, PBC6 - f, PBC7 - g, PBC8 - h, PBC9 - i, PBC 10 – j and PBC11 – k

Standardization of EPS Extraction

Many cyanobacterial species are surrounded by mucilaginous external layers such as capsule, sheath, mucilage, glycocalyx or slime. The exocellular mucilaginous material comprises polysaccharidic in nature. Extraction of these Capsular polysaccharides have been carried out by various chemical treatment followed by solvent precipitation. In order to get more quantity, standardization is more important.

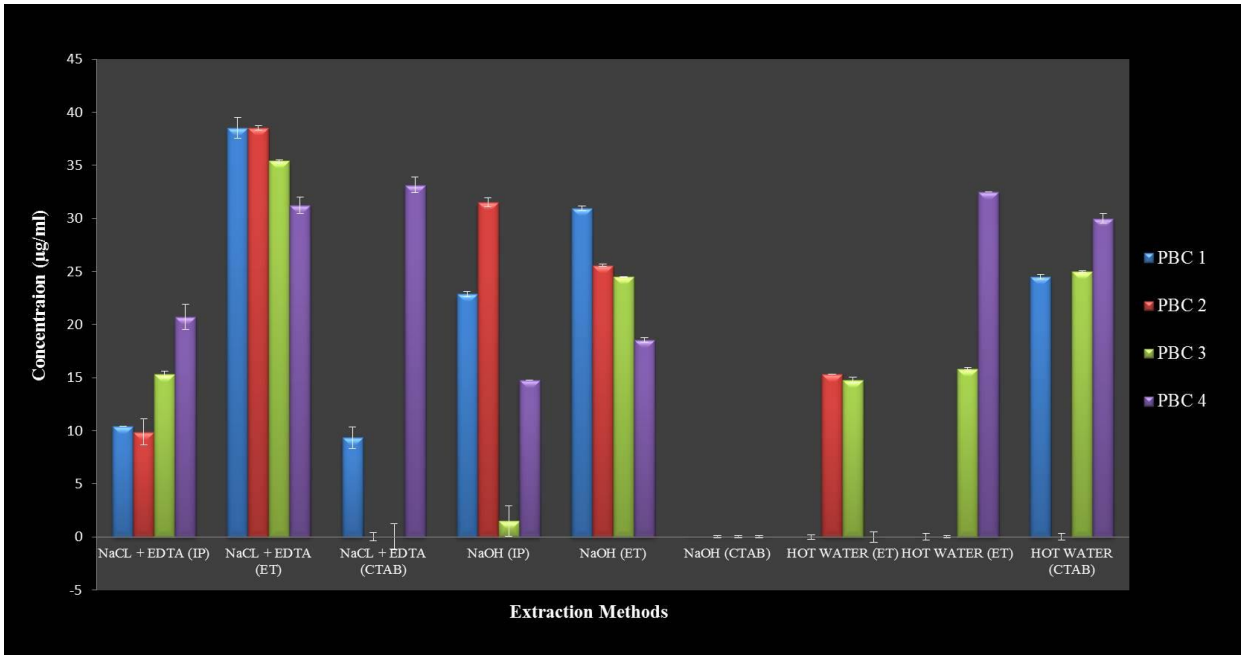

Figure 4: Standardization of CPS extraction using chemical and hot water treatment followed by solvent precipitation. (IP – Isopropanol, ET – Ethanol, CTAB – Cetyl Trimethyl Ammonium Bromide)

Organic solvents were used for the precipitation of neutral and acidic polysaccharides. CTAB alone could precipitate the acidic polysaccharide (Joana and Rosario, 1996). PBC 1 and PBC 2 showed the yield of 39µg mL^{-1} with extraction of NaCl + EDTA followed by ethanol precipitation. PBC 3 shows the yield of 40 µg mL^{-1} with hot water extraction followed by CTAB precipitation. PBC 4 shows the yield of 49 µg mL^{-1} with the hot water extraction followed by CTAB precipitation. Based on the results of figure 4, the four strains comprised of both neutral and acidic polysaccharides.

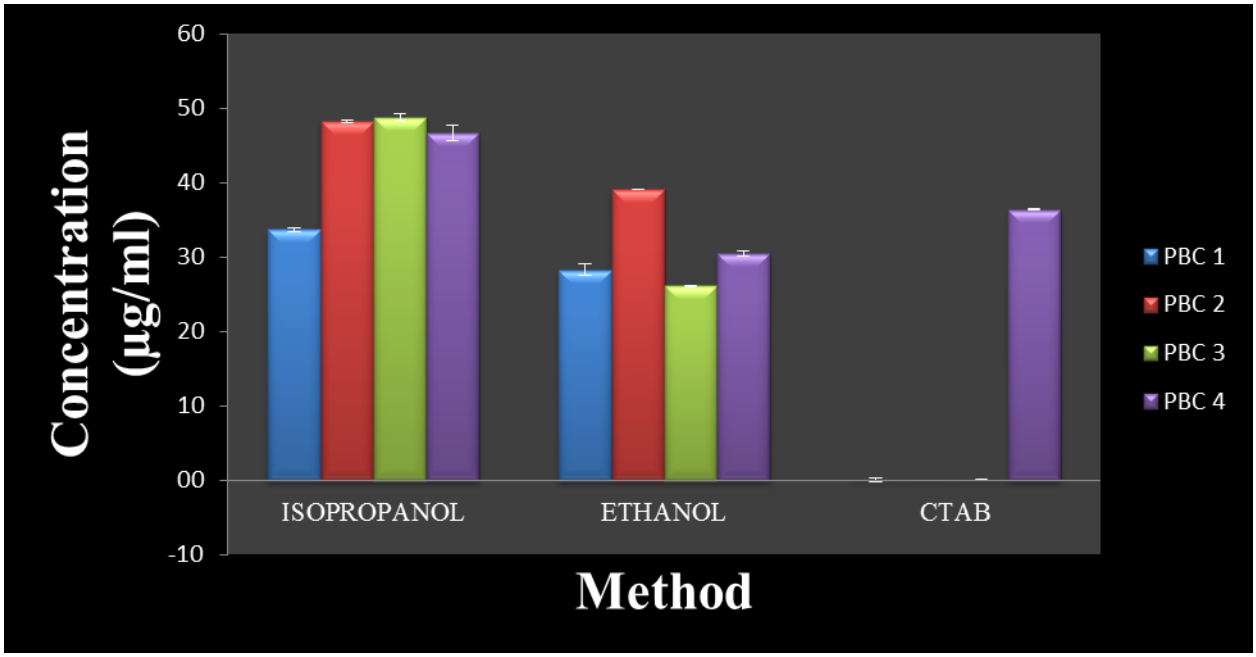

Figure 5: Standardization of Released Polysaccharide (RPS) precipitation using organic solvents and CTAB

Soluble polysaccharide or RPS is released by many cyanobacterial species into the media (Bold and Wenne, 1985). Organisms which produces large amount of RPS are biotechnologically important since it is easy to isolate released polysaccharides rather polysaccharides attached to the cell wall. Figure 5 shows the yield of precipitated RPS. PBC 1, PBC 2, PBC 3and PBC 4 RPS were well precipitated EPS in the equal volume of isopropanol compared to the ethanol in the yield of 34, 48, 49 and 47 µg mL^{-1} respectively.

Mass cultivation

Biomass and EPS of four strains were harvested in regular 6 days interval and fed-batch culture ran approximately 24 days. During the culturing phase of this study, cyanobacterial filaments showed the tendency which adhere glass walls to lead problem due to decreased active cell surfaces. This problem was anticipated because of the nature of the isolated strains streaming from well-structured benthic microbial communities (Richert, 2004). During culturing in the photo bioreactor, the adherence of filaments on glass walls was decreased by strong mechanical agitation.

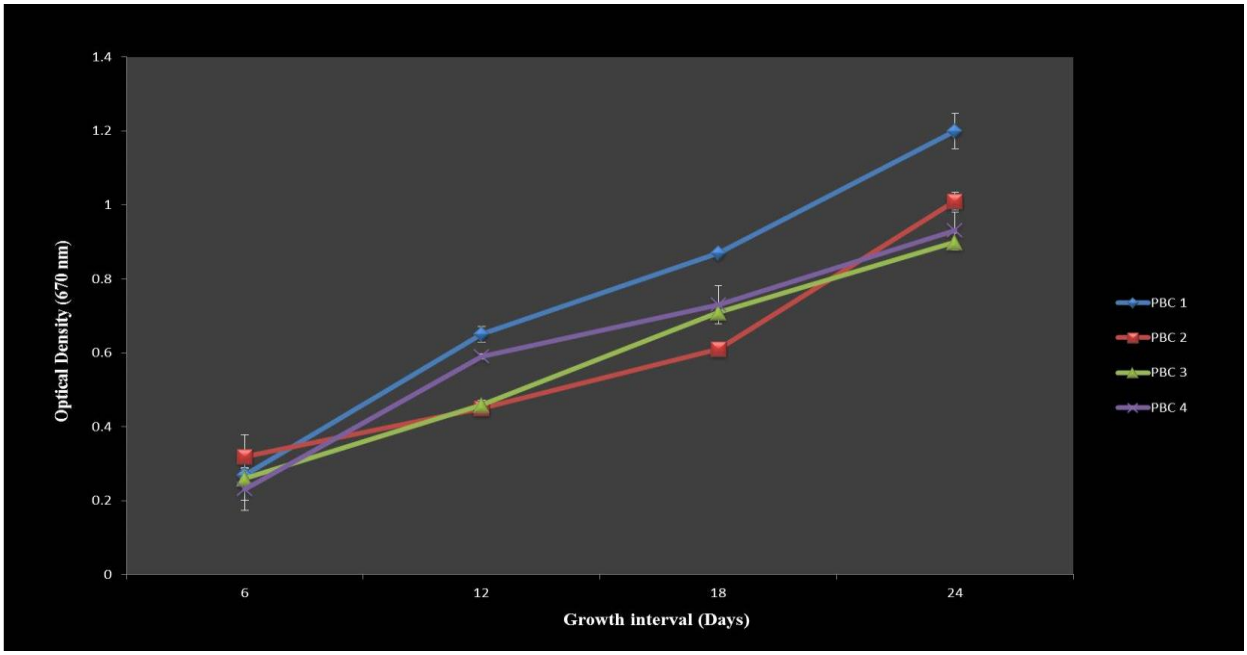

Figure 6: Cell density of PBC 1, PBC 2, PBC 3 and PBC *4* at 6 day's interval.

The maximum growth was obtained in the late exponential phase of all the cultures. The cell density of 7were 1.2, 1.0, 1.0 and 0.9 OD (Optical Density) at 670nm respectively. The production of large amounts of EPS has been suggested during the late exponential or stationary phase of growth (De Philippis and Vincenzini, 1998). So this cell density was enough to analyze the EPS production.

Carbohydrate Analysis

In order to determine the maximum production of polysaccharides such as RPS, CPS from back water isolates PBC 1, PBC 2, PBC 3and PBC 4, carbohydrate analysis was performed. The obtained results (figure 7 and 8) indicated that the concentration of EPS production was increased with regular time interval.

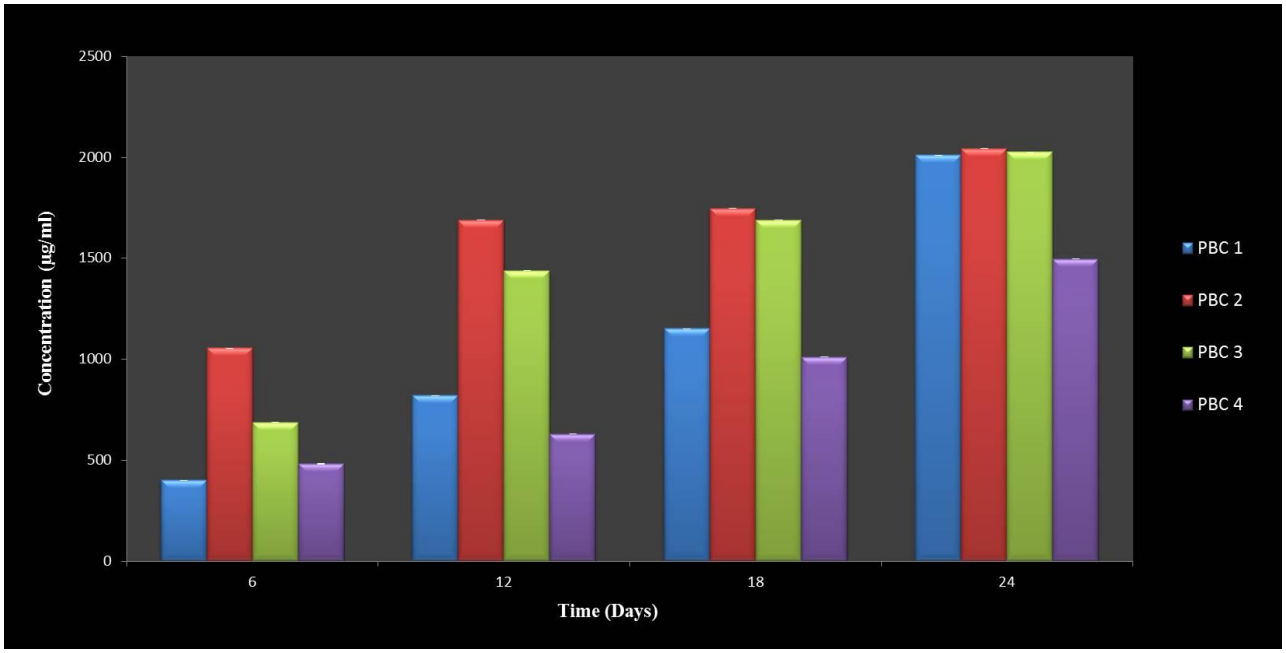

Figure 7: Released polysaccharide production in regular time interval

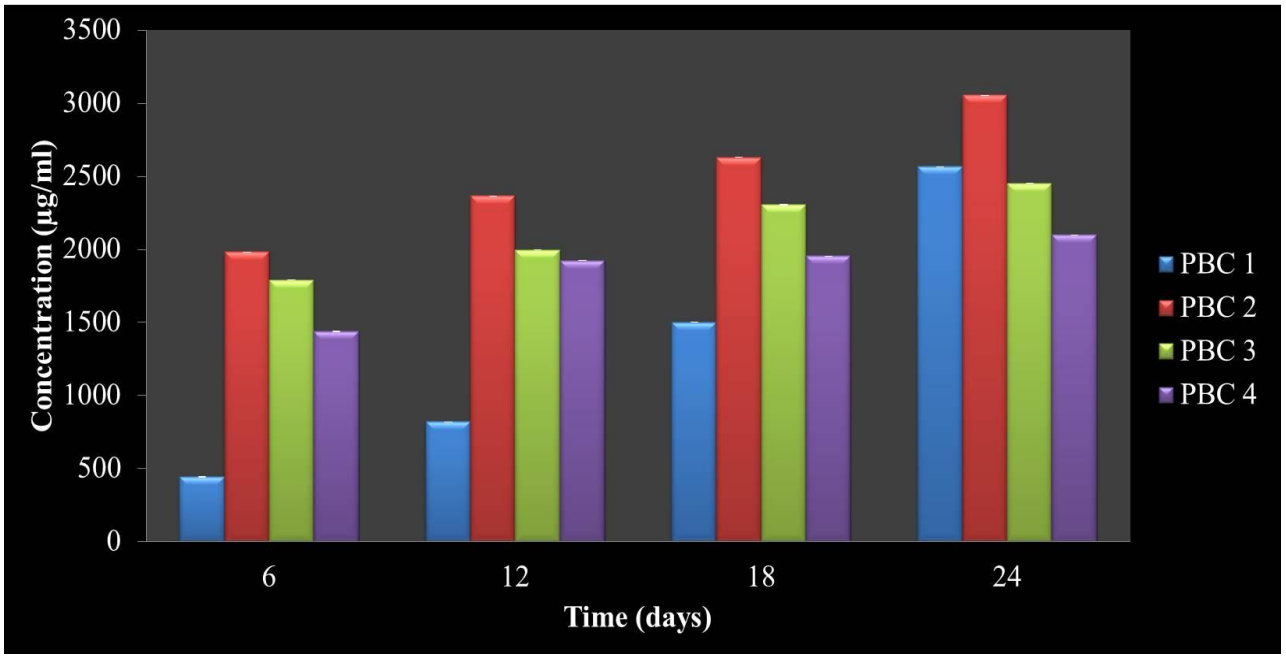

Figure 8: Capsular polysaccharide production in regular interval

Further, the dried EPS and CPS weight was calculated using modified protocol of Khattar et al., (2009). The maximum polysaccharide production was observed in unicellular strain PBC 4, 66mg L^{-1} for RPS and 35mg L^{-1} for CPS compared to the filamentous strain PBC 1, 53mg L^{-1} for RPS and 20mg L^{-1} for CPS. PBC 2 was produced 45mg L^{-1} for RPS and 19mg L^{-1} for CPS and the lowest polysaccharide production was observed in PBC 3, 27mg L^{-}

[1] for RPS and 10mg L^{-1} for CPS. Unicellular form of cyanobacterial species was found to produce higher amount of polysaccharide when compared to filamentous form.

Polysaccharide characterization

Visualization of Exopolysaccharide production

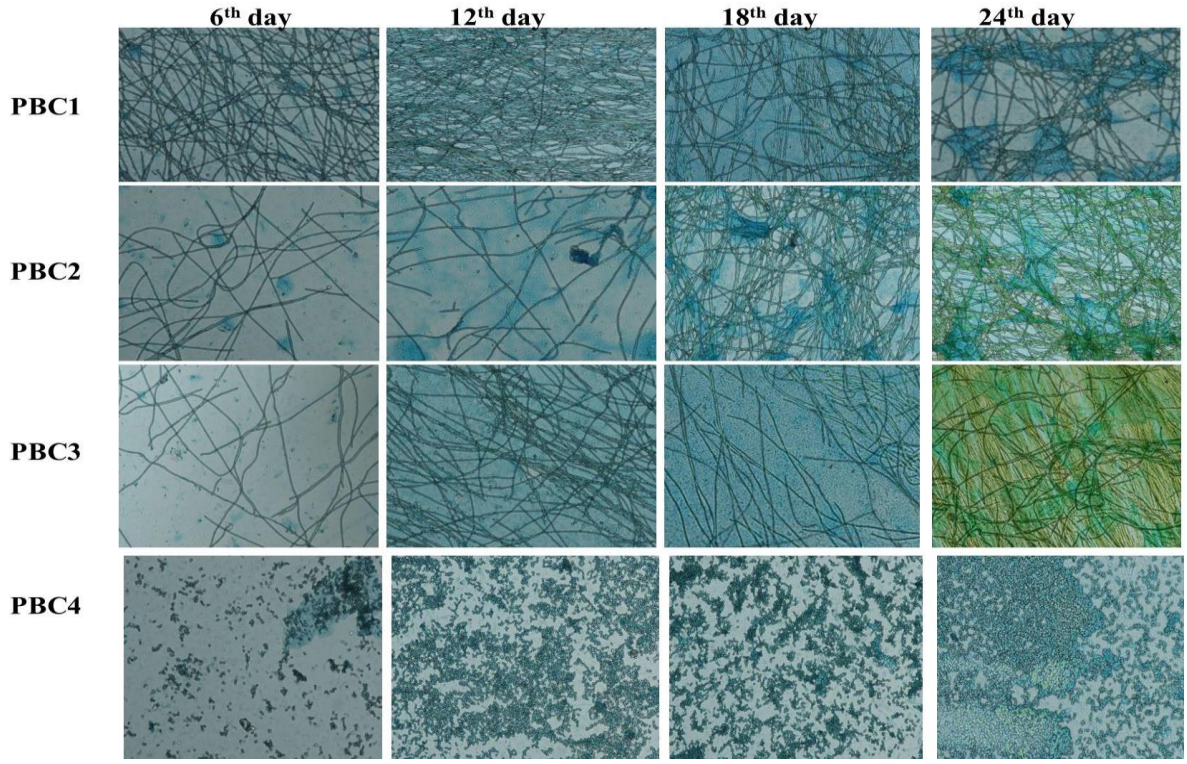

Figure 9: Light micrographs of cyanobacterial strains after staining with Alcian Blue

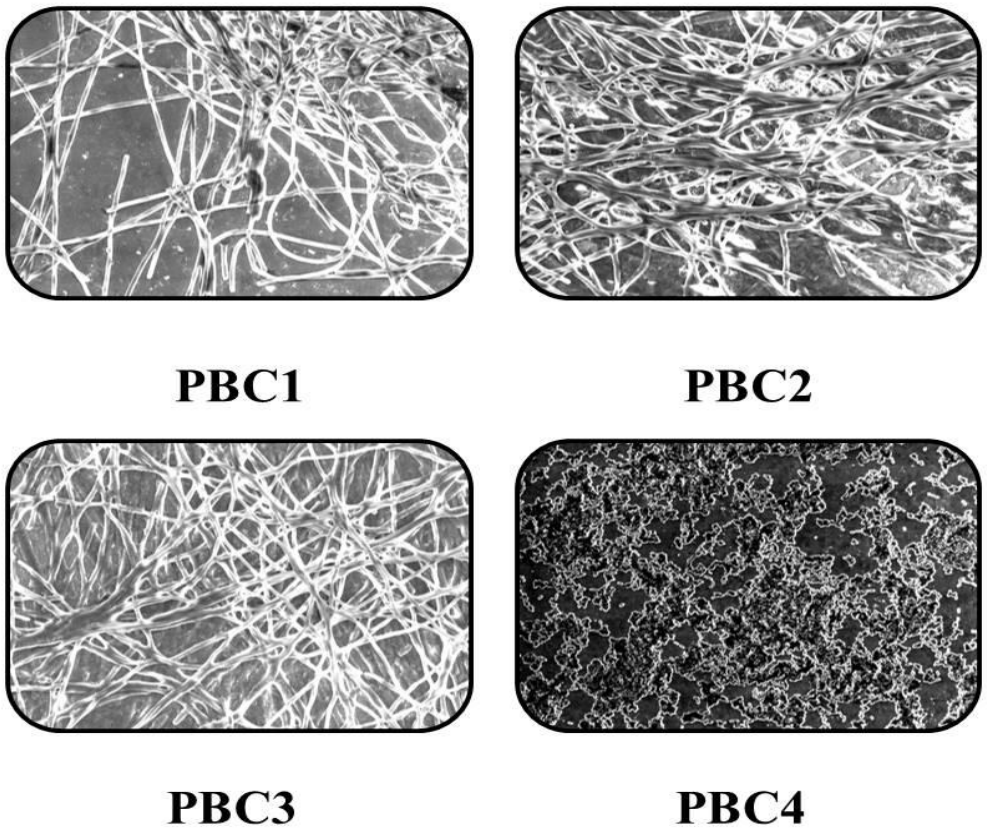

Figure 10: visualization of EPS production under SEM

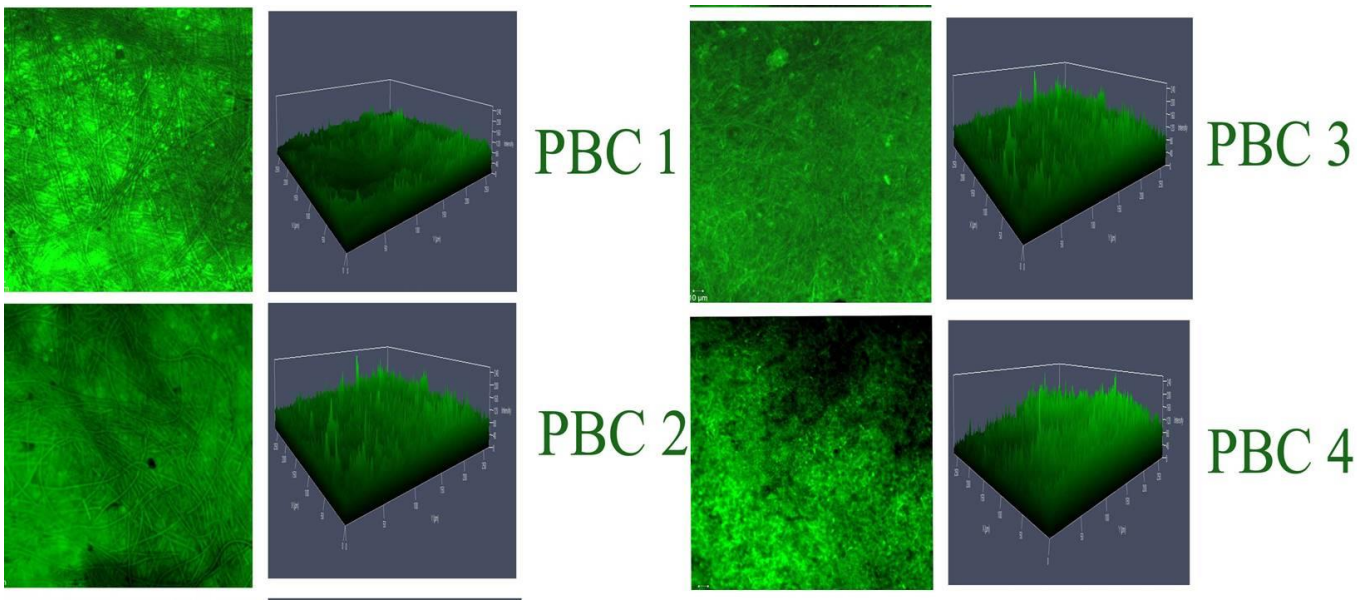

Figure 11: Visualization of EPS under Confocal Laser Scanning Microscopy

` Light microscope observations (figure 9) after staining with alcian blue showed a dense blue coloration surrounding the cells of all the four cyanobacterial strains. The dye bounded to the material released into the medium, suggesting the presence of anionic compounds, both in the layer tightly attached to the cells and in the solubilized RPS found in the culture medium.The Light microscopic picture also support the EPS production was increased during the regular time interval. SEM (figure 9) image could support the presence of EPS surrounding the cells. CLSM (figure 10) of four cyanobacterial strains were indicated the formation of biofilm during their growth. EPS mainly consist of exopolysaccharides, proteins, nucleic acids and lipids that form a three-dimensional polymer network attaching the biofilm to surfaces and holding cells in close proximity resulting in a synergistic community (Flemming and Wingender 2010). The four strains grown in the plastic trays with aerator prototype showed an initial uneven adhesion to the substratum after which the spaces eventually filled in as the biofilm developed forming a homogenous covering of the entire growing surface.

Infrared spectroscopy of dried EPS

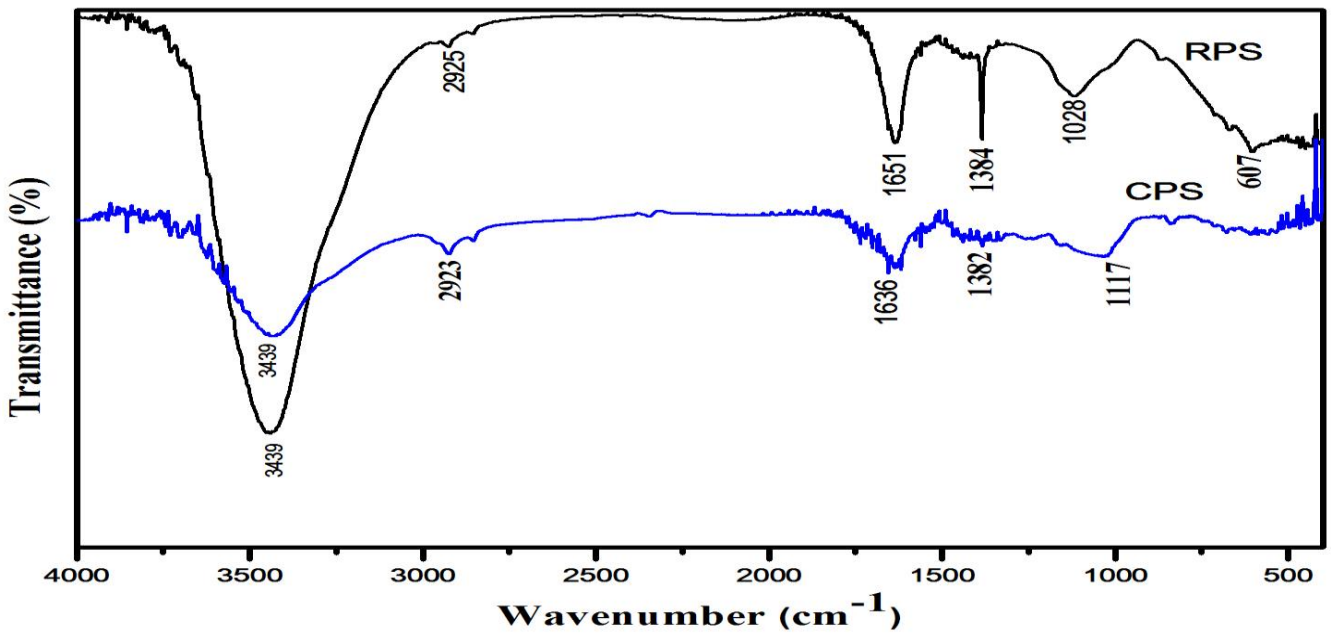

Figure 12: Comparative FT-IR spectra of RPS and CPS from PBC 1

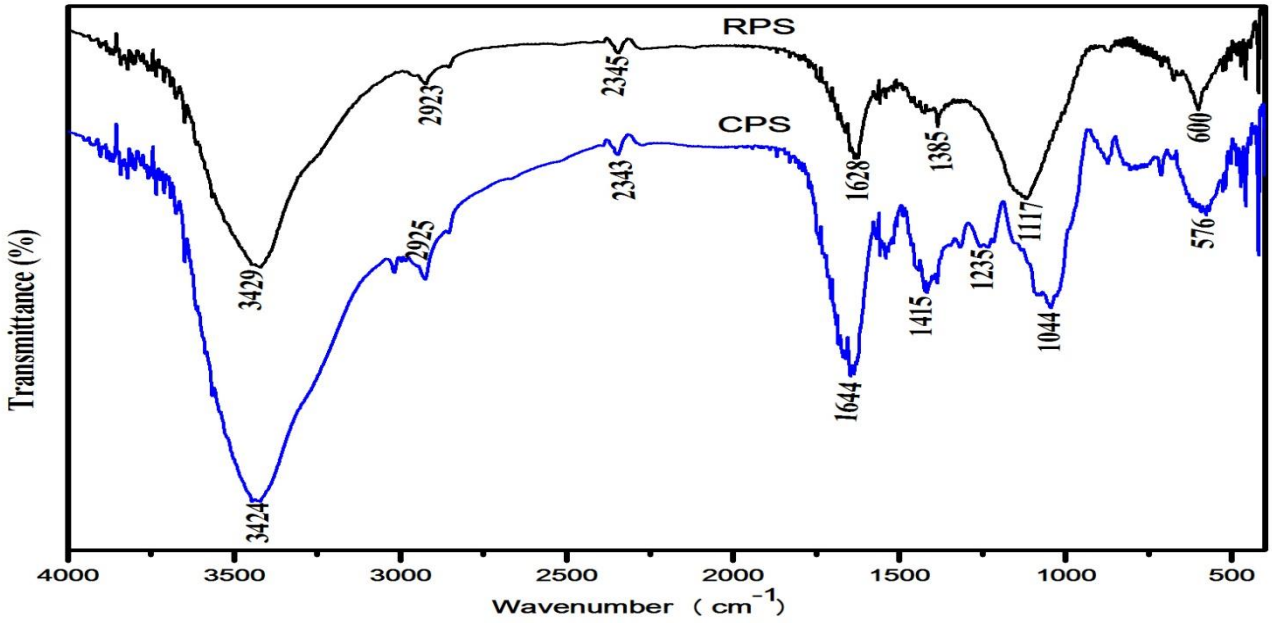

Figure 13: Comparative FT-IR spectra of RPS and CPS from PBC 2

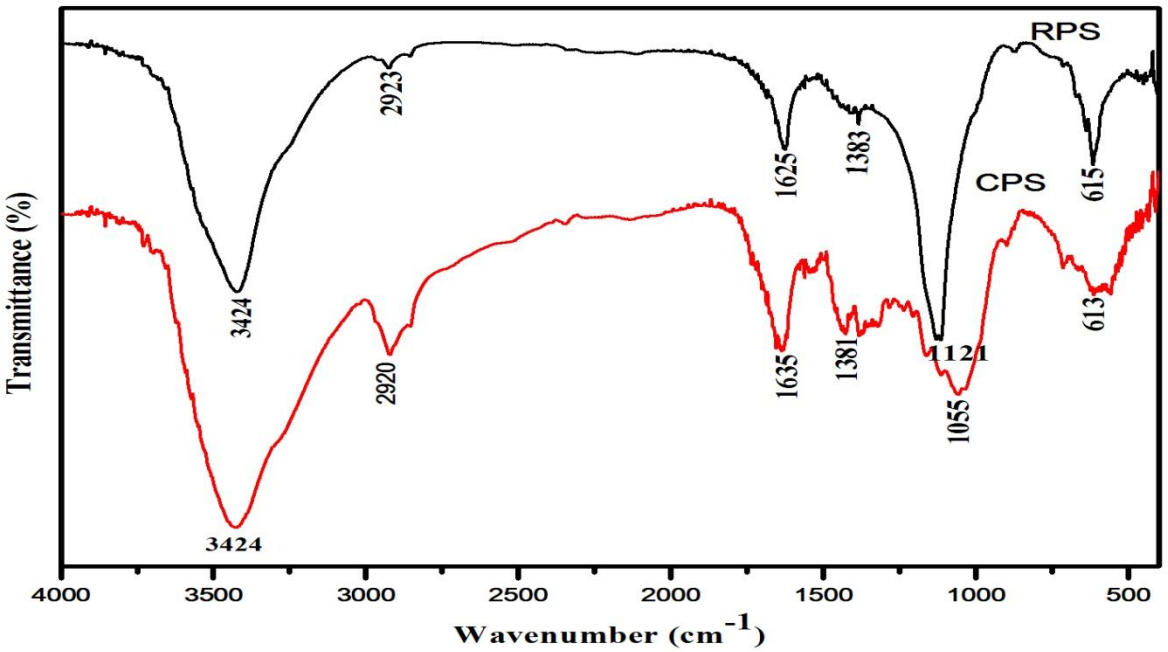

Figure 14: Comparative FT-IR spectra of RPS and CPS from PBC 3

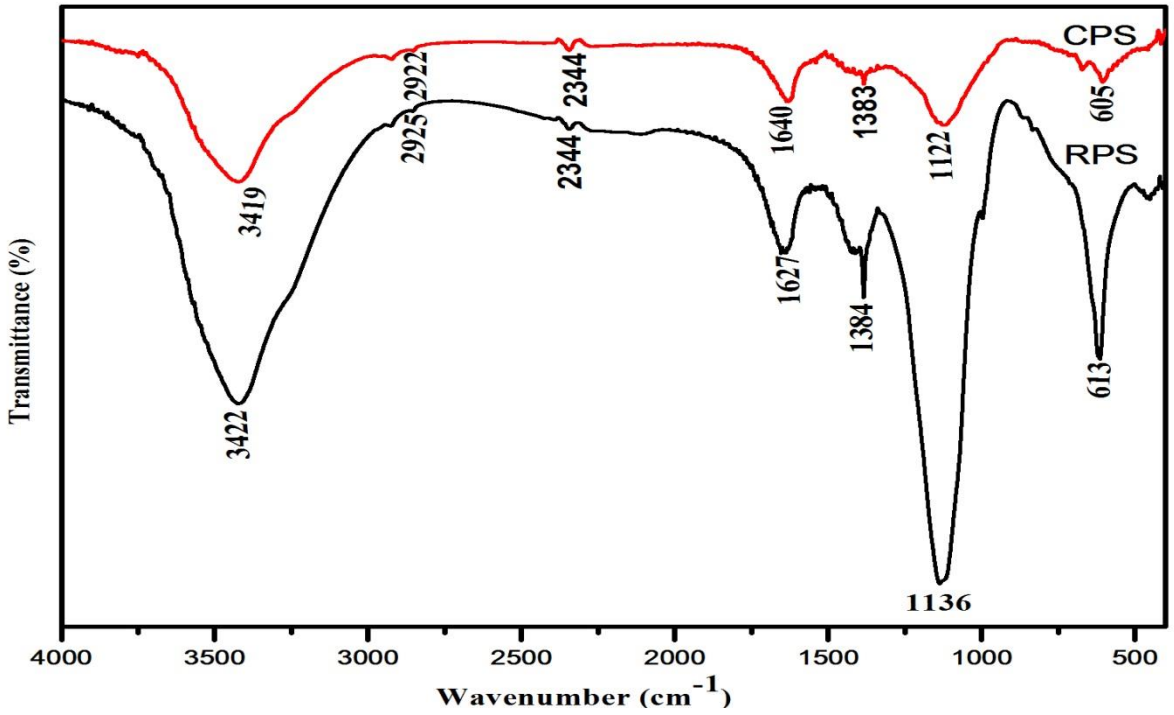

Figure 15: Comparative FT-IR spectra of RPS and CPS from PBC 4

Variation in stretching and bending modes of vibration with single functional group is normally coupled with the vibration of adjacent group as well as with the number of substitution/s taking place on the molecule itself. This leads to the shifting or overlapping of the peaks of two or more functional group in the same region of IR spectrum (Silverstein and Webster, 1998; de Sousa, 2004).

Comparative IR spectrums of RPS and CPS obtained from four cyanobacterial are shown in figure 12, 13, 14 and 15 which shows more complex pattern of peaks from 2950 to 1200 cm^{-1}. From the FT-IR spectrum of the polysaccharide of four cyanobacterial strains, functional group characteristics were analyzed, a broad spectrum from 3,500 to 3,200 cm^{-1} is due to OH stretching vibrations, a strong absorption near 2,920 to 2,950 cm^{-1} is assigned to aliphatic C–H stretching vibrations. Strong stretching vibration of carboxylate group appeared at 1,620 to 1.660 cm^{-1}, absorption for C–H bending of aliphatic CH2 group observed at 1,300 to 1,400 cm^{-1}.

The C=O absorption of uronic acids occurred at 1,620 to 1,651 cm^{-1}. A medium intense peak at 1,040 to 1,050 cm^{-1} might be due to C–S stretching, the absence of S=O stretching near 1,250 cm-1 indicates that sulphate group may be present in its salted form. Polysaccharide possessing sulphate group is reported to have antiviral properties (Witvrouw and De Clercq 1997; Ono et al. 2003). The appearance of several peaks at 850 to 920 cm^{-1} indicated the a-configuration of D-glucan (Peng *et al.*, 2005). The relatively strong absorption peak at around 1,650 cm-1 and the weak one at around 1,250 cm-1 also indicated the characteristic IR absorption of polysaccharide (Shi *et al.*, 2007).

An important role in the metal sorbing capacity of the EPS-producing cyanobacteria has been attributed to the negative charges generally present in the cyanobacterial EPSs (Pereira et al. 2009). The cyanobacterial exopolysaccharides are known to interact strongly with cations. In the present study, FT-IR studies showed that the functional groups such as carboxyl, hydroxyl and sulphate groups mainly owing to the high content of uronic acids present in our cyanobacterial exopolysaccharides. These functional groups of exopolysaccharides seem to be the chelation of Na^{+} from Saline soil.

Optimization of photoperiods and pH for polysaccharide production

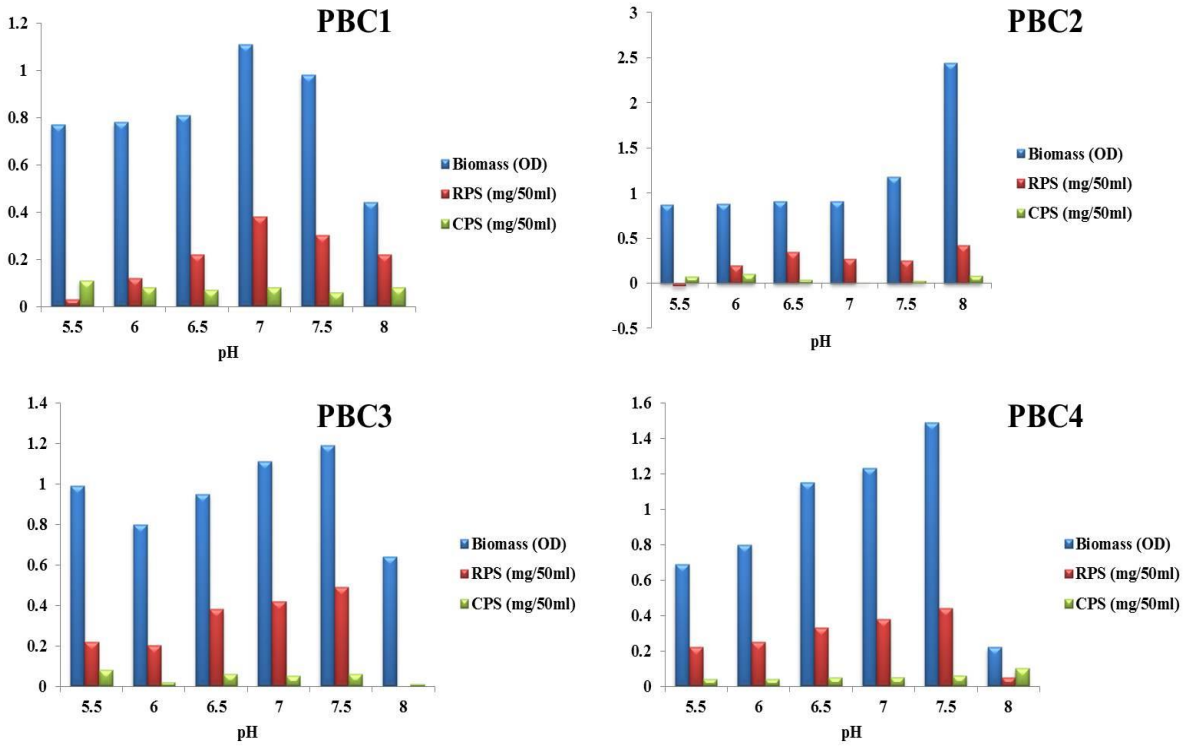

Figure 16: Production of RPS and CPS respective to the biomass concentration in various pH

The biomass concentration was increased at pH 7.5 except PBC 2. Figure165 shows the yield of precipitated RPS respect to the biomass concentration. PBC 1, PBC 2, PBC 3and PBC 4 RPS were well precipitated EPS in the equal volume of isopropanol in the yield of 38, 15, 49 and 44 µg/50ml respectively. The result shows that during biomass production RPS was produced simultaneously. pH 7.5 was optimum for the all the culture which is isolated from the backwater (Selvakumar and Sundararaman, 2009).

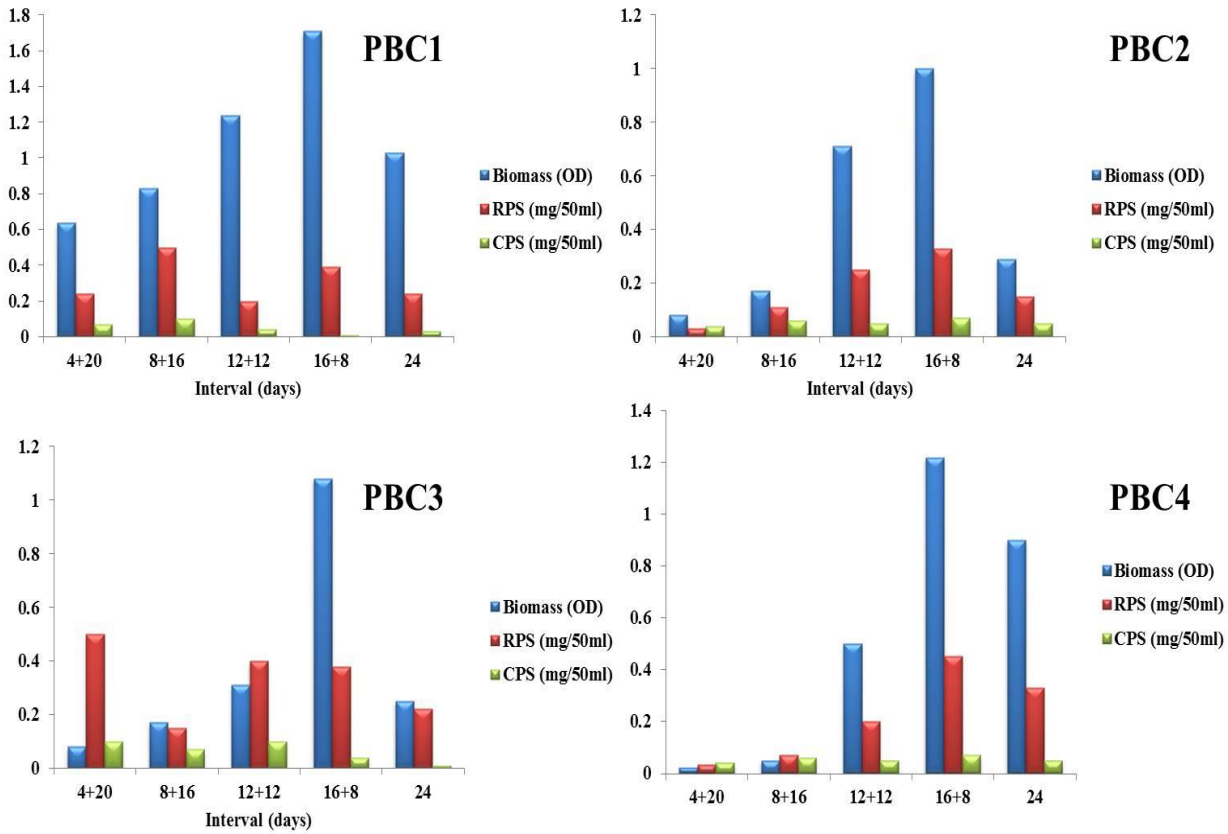

Figure 17: Production of RPS and CPS respective to the biomass concentration in various photo periods

An estimation of the light photon demand of photoautotrophic cyanobacteria was simplified by consideration of the anabolic demands for production of cellular carbohydrate (Das et al., 2010). The biomass concentration was increased at 16+8 hours exposure of light. Figure 17 shows the yield of precipitated RPS respect to the biomass concentration. PBC 1, PBC 2, PBC 3and PBC 4 RPS were well precipitated EPS in the equal volume of isopropanol in the yield of 39, 33, 38 and 45 µg/50ml respectively. The result shows that during biomass production RPS was produced simultaneously. 16+8 hours exposure of light was optimum for the all the culture which is isolated from the backwater.

Seed germination experiment with and without NaCl

Sample	Seed germination (%)	Root length (cm)	Shoot length (cm)
PC- PCB 1 1mg	100	8.3	4.2
PC- PCB 2 1mg	100	12.3	4.5
PC- PCB3 1mg	100	10.7	4.8
PC- PCB 4 1mg	100	12.5	4.5
PC- PCB 1 2mg	100	9.2	5.2
PC- PCB 2 2mg	100	10.6	5.5
PC- PCB3 2mg	100	10.4	5.0
PC- PCB 4 2mg	100	14.7	5.2
PC- PCB 1 3mg	100	10.8	5.6
PC- PCB 2 3mg	100	10.5	6.0
PC- PCB3 3mg	100	13.3	5.1
PC- PCB 4 3mg	100	15.1	5.6
PC- PCB 1 4mg	100	11.9	5.7
PC- PCB 2 4mg	100	11.2	6.2
PC- PCB3 4mg	100	14.2	5.7
PC- PCB 4 4mg	100	20.1	6.3

NC- 0.05M	100	8.1	4.0
NC- 0.1M	100	7.5	4.2
NC- 1M	-	-	-
NC- 2M	-	-	-
PBC1 -0.1M+2mg	100	7.0	4.3
PBC2 -0.1M+2mg	100	9.2	4.5
PBC3 -0.1M+2mg	100	4.1	4.3
PBC4 -0.1M+2mg	100	5.1	4.1
PBC1 -1M+2mg	-	-	-
PBC2 -1M+2mg	-	-	-
PBC3 -1M+2mg	-	-	-
PBC4 -1M+2mg	-	-	-
PBC1 -2M+2mg	-	-	-
PBC2 -2M+2mg	-	-	-
PBC3 -2M+2mg	-	-	-
PBC4 -2M+2mg	-	-	-
PBC1 -0.1M+4mg	100	7.5	4.5
PBC2 -0.1M+4mg	100	11.1	6.0

PBC3 -0.1M+4mg	100	5.0	5.3
PBC4 -0.1M+4mg	100	6.5	4.5
PBC1 -1M+4mg	-	-	-
PBC2 -1M+4mg	-	-	-
PBC3 -1M+4mg	-	-	-
PBC4 -1M+4mg	**100**	**11.5**	**3.5**
PBC1 -2M+4mg	-	-	-
PBC2 -2M+4mg	-	-	-
PBC3 -2M+4mg	-	-	-
PBC4 -2M+4mg	-	-	-

Table 1: Effect of Cyanobacterial EPS with and without NaCl on seed germination. (PC- Positive control, NC- Negative control)

The various concentrations of exopolysaccharides of cyanobacterial strains such as (1mg, 2mg, 3mg, 4mg) showed the 100 percent germination of seeds. The highest seed germination was observed in PCB 4 with 4mg concentration of EPS rather than other strains. The length of root and shoot were significantly increased in treatment with EPS.

The seeds were treated with (0.1M , 1M and 2M) different concentrations of NaCl and (2mg , 4mg) EPS in order to observe the tolerance of seeds to thrive in salt condition. It showed that 100 percent germination in 0.1M concentration. The length of root and shoot of

PCB4 increased greatly when compared to other strains. Also, PCB 4 EPS (4mg) was exhibited salinity tolerance in 1M NaCl concentration resulted in root length 11.5 cm and shoot length 3.5 cm (Table 1).

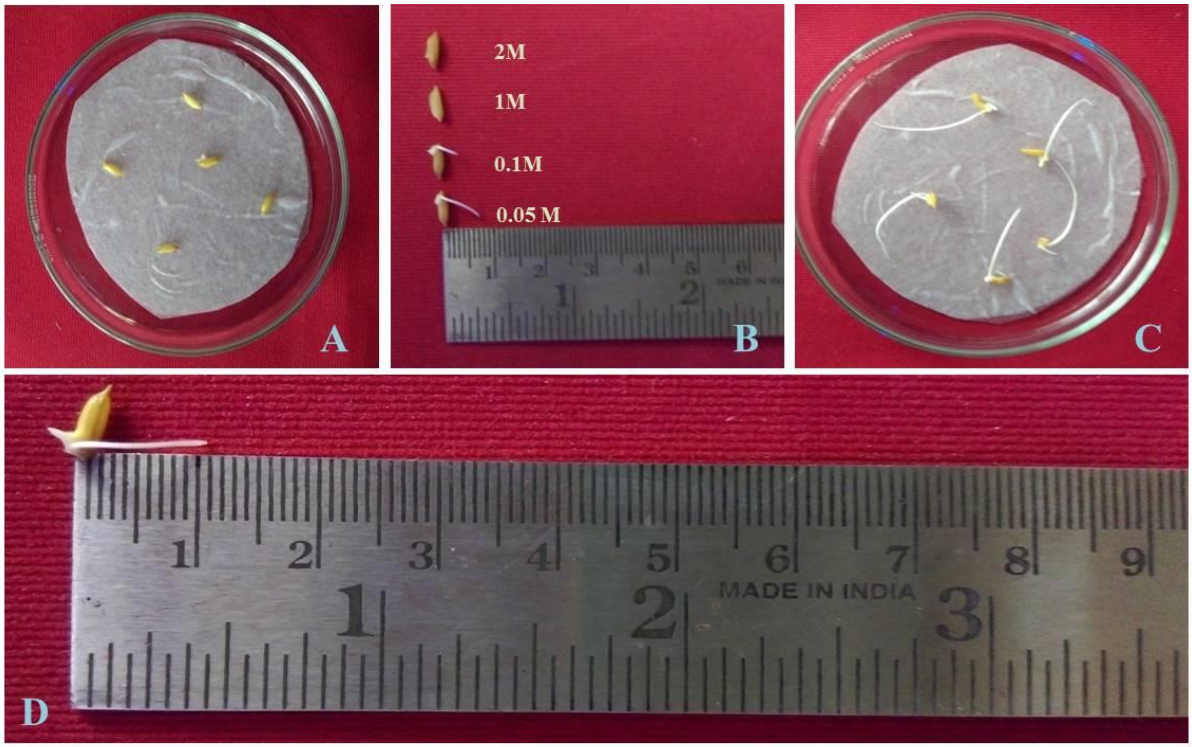

Figure 18: Photograph of Seed germination experiment with Cyanobacterial EPS. A- 1st day of seed germination. B- NaCl treated seeds. C- 4th day of germination seeds treated with PBC 4 EPS (4mg) and D- Scale bar.

Estimation of Chlorophyll

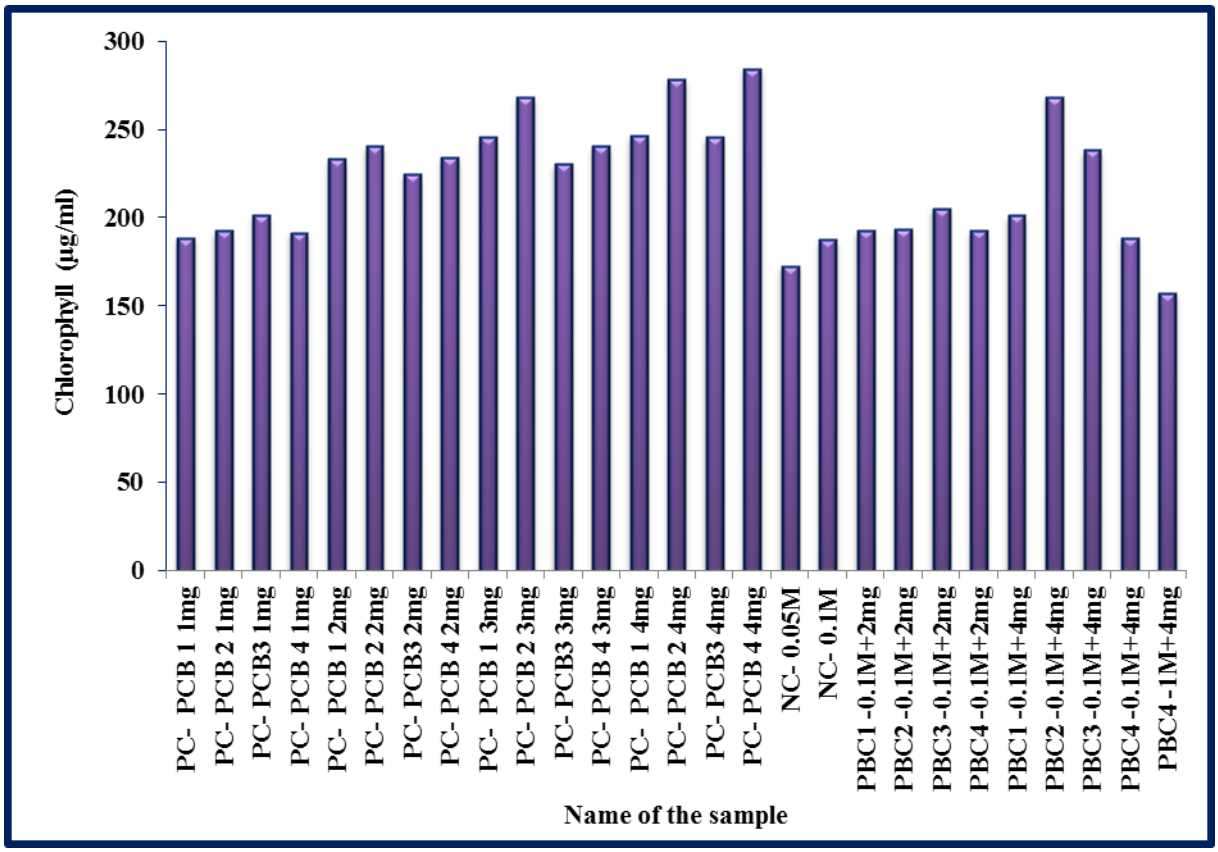

Figure 19: Estimation of Chlorophyll

Figure 19 represents the EPS produced by these four isolated strains has increased chlorophyll content in which PCB 4 contains larger production of chlorophyll 255µg/ml at 4mg concentration of EPS. Moreover, the culture PCB 2 treated with 0.1 M NaCl and 4mg EPS has shown to contain 250µg/ml of chlorophyll pigment which was greater than other culture strains.

Preparation of the Algal filtrate

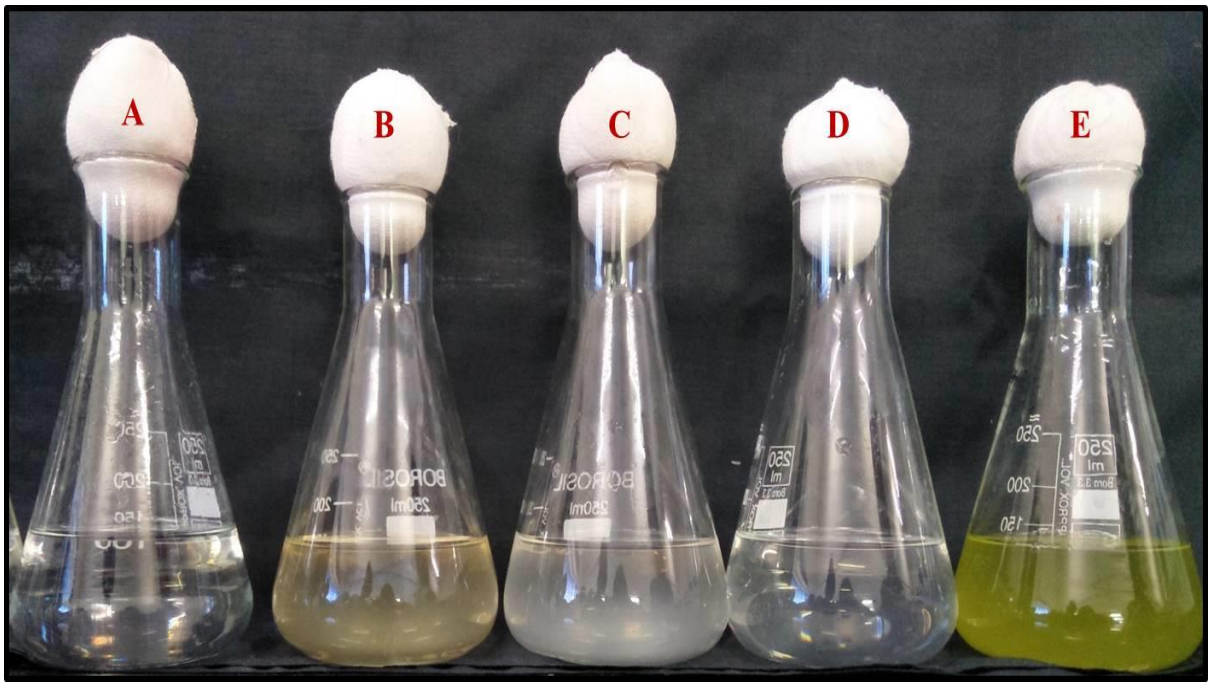

Figure 20: Preparation of Algal filtrate A – control, B – PCB 1 , C – PCB 2 , D – PCB 3, E – PCB 4.

The algal filtrate was prepared in 250ml conical flasks with the amount of 100ml filtrate and store at 4^0C for seed germination studies.

Seed germination experiment for algal filtrate

Sample	Seed germination (%)	Root length (cm)		Shoot length (cm)	
		4th day	7th day	4th day	7th day
Control	80	3	4	2.6	4.3
PBC 1	100	2.7	4	2.7	5
PBC 2	90	3.3	6.2	2.3	5.5

PBC 3	**90**	**4.2**	**6.2**	**1.8**	**6.9**
PBC 4	100	3.5	5.1	2.3	6.7
PBC1 - 0.1M+2mg	100	3.2	5.1	1.9	5.2
PBC2 - 0.1M+2mg	100	3.1	5.3	2.2	5.3
PBC3 - 0.1M+2mg	100	2.9	4.6	2.5	5.4
PBC4 - 0.1M+2mg	100	2.6	4.2	2.3	5.2
PBC1 - 1M+2mg	-	-	-	-	-
PBC2 - 1M+2mg	-	-	-	-	-
PBC3 - 1M+2mg	-	-	-	-	-
PBC4 - 1M+2mg	-	-	-	-	-
PBC1 - 0.1M+4mg	100	3.6	5.0	2.1	4.5
PBC2 - 0.1M+4mg	100	3.2	5.1	2.0	5.0
PBC3 - 0.1M+4mg	100	4	6.0	2.3	5.8
PBC4 - 0.1M+4mg	100	3.5	4.2	2.5	5.3
PBC1 - 1M+4mg	-	-	-	-	-
PBC2 - 1M+4mg	-	-	-	-	-
PBC3 - 1M+4mg	-	-	-	-	-
PBC4 - 1M+4mg	-	-	-	-	-

Table 2: Seed germination experiment for Algal filtrate with and without NaCl

100% seed germination frequency was observed in all the control, cell filtrate treated and cell filtrate with 0.1M NaCl except cell filtrate with 1M NaCl. The lowest seed germination frequency was observed in control (80%). The shoot length of rice seedlings was highly significant increase in response to all culture filtrates with in relation to control (Figure 21). The maximum increase in shoot length and root length was observed in PBC 3 in range of 6.2cm and 6.9cm. The lowest shoot length and root length was observed in PBC 1 in range of 4cm and 5cm. These results were further confirmed by lab culture experiment (figure 22).

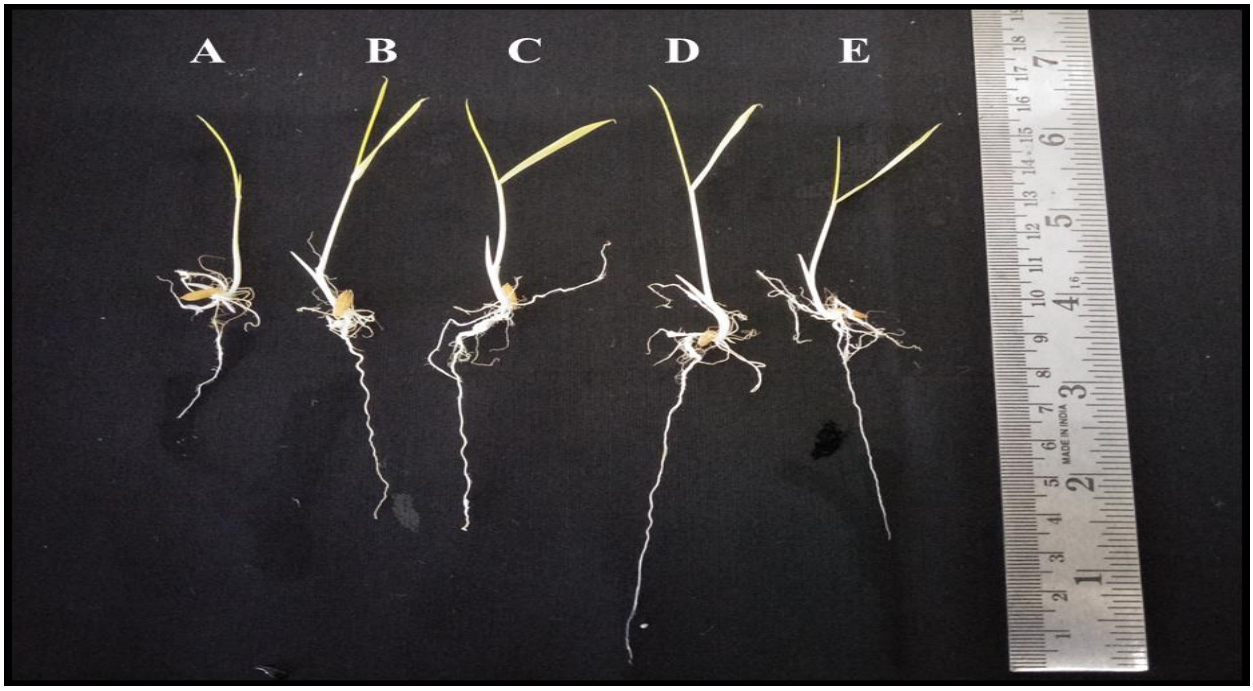

Figure 21: Photograph of Seed germination experiment for Algal filtrate.

A- Control, B- PBC1, C- PBC2, D- PBC3, E- PBC4

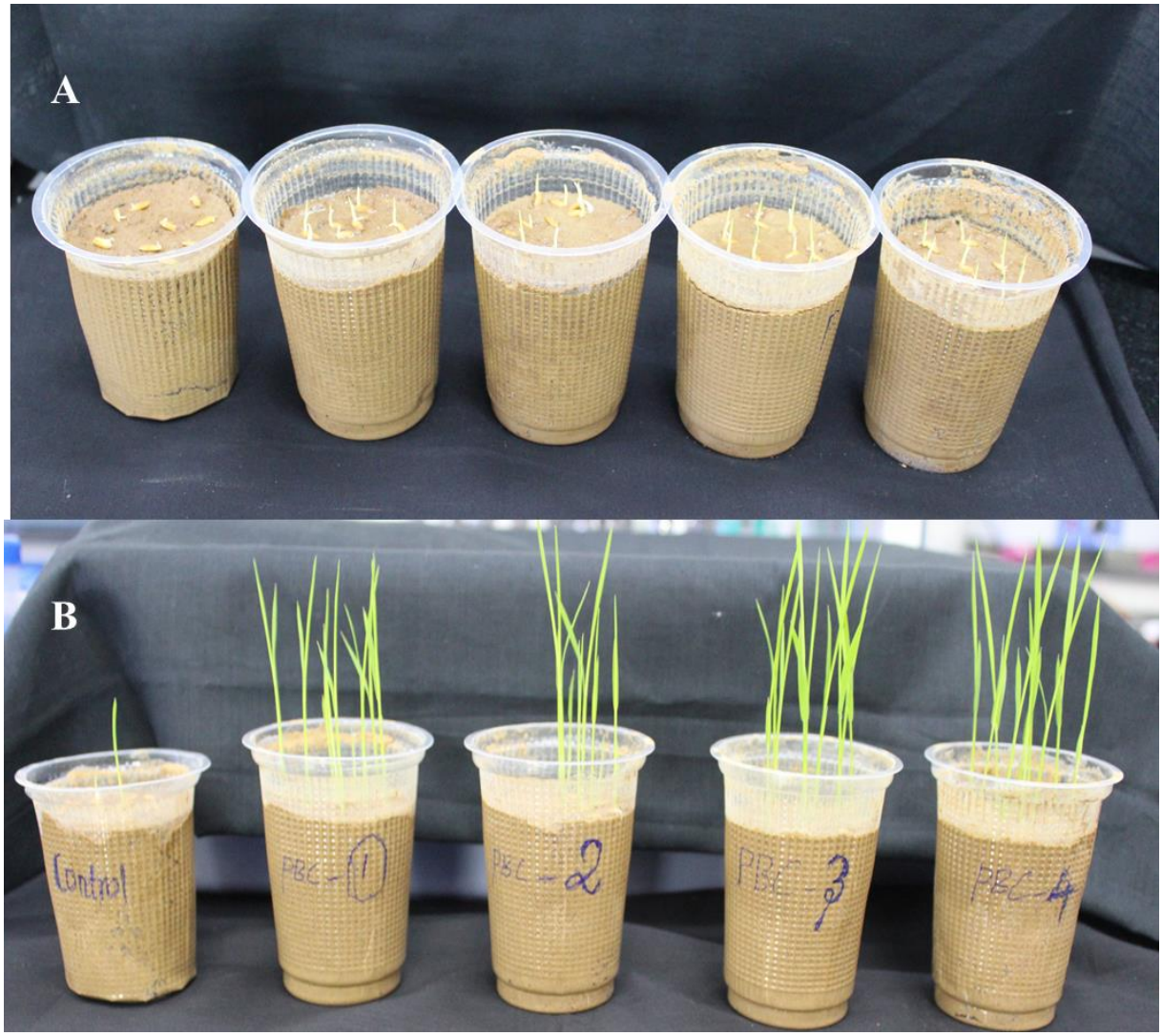

Figure 22: Lab culture experiment for Algal filtrate.

A- 2nd day of germination and B- 7th day of germination

Exopolysaccharides producing four strains were isolated from the Kattumavadi backwater of Palk Strait Region, Tamilnadu, India. The salinity of the backwater changes according to the rate of fresh water flow. Cyanobacteria which are present in the backwater region have the ability to tolerate wide range of salinity. In this region, some cyanobacterial species are capable of excreting polysaccharides, proteins and plant growth substances.

Germination is a vital phase in the life cycle of plants, which aids the embryo to endure the period between seed maturation and seedling establishment. There are many factors affecting on seed germination such as salinity, temperature, moisture and light intensity. Thus application of cyanobacterial exopolysaccharides or extracts to seeds can

significantly improve seed germination and seedling growth in different crops. Thus the present study shows that cyanobacteria boost up germination and seedling growth of the crop species, indicating growth stimulating factors present in the EPS. The EPS seems to play a crucial role by binding the hazardous Na^+, thereby alleviating the adverse effects of salts on crops.

The shoot length and root length were significantly increased in treatment with cyanobacteria. The chlorophyll content of shoot which was grown also increased significantly. The growth parameters of rice indicated that accumulation of soluble salts in soil affected the rice growth whereas soil treated with PBC1, PBC2, PBC3 and PBC4 influenced better response to plant growth. The treatment of saline alkali soil with backwater species significantly reduced the exchangeable sodium percentage with treatment of free and immobilized cyanobacteria. Cyanobacteria which trapped the sodium temporarily and also acclimatized to grow in saline alkali soil. In present study, PCB 4 EPS (4mg) was exhibited salinity tolerance in 1M NaCl concentration resulted in root length 11.5 cm and shoot length 3.5 cm. Moreover, PCB 2 and PCB 4 were contained more amount of chlorophyll.

These organisms can be used to reclaim alkaline soils because they form a thick stratum on the surface of the soil during the rainy season and the winter months. The algal material incorporated in the soil conserves organic C and N, and organic P as well as moisture, and converts Na^+ clay to Ca_2^+ clay. Accumulation of K^+, exclusion of Na^+ and maintenance of low intracellular Na^+ levels, synthesis of carbohydrates, polyols, amino acids and quaternary amines for osmoregulation and other adaptations of metabolism are principal features associated with and contributing to the salt tolerance in cyanobacteria. Extracellular mucopolysaccharides chelate significant amounts of sodium. Intracellular sodium exists as a free cation and is not incorporated into any biomolecule, especially proteins.

It was evidenced from the obtained results that the cyanobacterial culture filtrates contain variable amount of soluble carbohydrates and proteins in addition to soluble phosphorus. The presence of these compounds in the culture supernatant could participate in the stimulation of seed germination and growth parameters of plant seedlings. In agricultural soil, cyanobacteria, a main constituent of the natural microbiota play a crucial task in improving soil fertility and increasing crop productivity. The presoaking of plant seeds in the cyanobacterial exudates could be an economical and environment friendly strategy to reduce the utilizing of the expensive chemical fertilizers. In present investigation, 100% seed

germination frequency was observed in PBC1 and PBC4 in response to all culture filtrate. The maximum increase in shoot length and root length was observed in PBC 3 in range of 6.2cm and 6.9cm.

Molecular characterization

EPS producing cyanobacterial strains PBC1, PBC2, PBC3 and PBC4 (Fig. 23) were well attached to the surface of the plastic trays and ability to form biofilms on the surface. PBC1, PBC2 and PBC3 were belongs to the filamentous group and PBC4 belongs to the coccoid group. These cyanobacterial strains were successfully identified through partial 16S rRNA gene sequences. PBC1, PBC2, PBC3 and PBC4 were identified as *Phormidium lucidum* ALU PBC1, *Leptolyngbya valderiana* ALU PBC2, *Plectonema terebrans* ALU PBC3 and *Cyanobacterium aponinum* ALU PBC4 through BLAST analysis by sequence similarity. The evolutionary history of these strains inferred by using the Maximum Likelyhood Method based on the Tamura-Nei model (Tamura and Nei, 1993).

Strain Name	Name	Banklet No	Accession No
PBC 1	*Phormidium lucidum* ALU PBC 1	Banklet 1947432 seq1	KX780066
PBC2	*Leptolyngbya valderiana* ALU PBC 2	Banklet 1950053 seq1	KX816335
PBC3	*Plectonema terebrans* ALU PBC 3	Banklet 1953182 seq1	KX853084
PBC4	*Cyanobacterium aponinum* ALU PBC 4	Banklet 1953183 seq1	KX853085

Figure 23: GenBank submission of isolated EPS producing strains.

Summary Enclosure 2

The present research work was carried to isolate *Phormidium lucidum* ALU PBC1, *Leptolyngbya valderiana* ALU PBC2, *Plectonema terebrans* ALU PBC3 and *Cyanobacterium aponinum* ALU PBC4 from Kattumavadi backwater of Palk Strait Region, Tamilnadu, India. These strains were produced higher amount of Exopolysaccharide on 24th day of incubation. Light microscope observations after staining with Alcian blue showed a dense blue coloration surrounding the cells of all the four cyanobacterial strains because of the EPS production. The advantage of this approach probably lies in the apparent ability of cyanobacterial polysaccharides to 'chelate' considerable amounts of sodium and temporarily immobilize the excess sodium. It was cleared from these studies that the beneficial effect of cyanobacteria on the crop plants may not be restricted to their role in the fixation of atmospheric nitrogen. They can stimulate the growth and development of plants lying in their habitat via different approaches including biofertilizers, biological control, soil conditioners, biosorption of heavy metals or by delivering of various biologically active substances. The amount of EPS produced by cyanobacteria and the culture filtrate enhanced the germination of seed with 0.1M NaCl concentration, which promotes the growth of rice and increased the root, shoot, chlorophyll content of plant. Observation of present investigation showed that backwater cyanobacterial strains reclaimed the affected soil and improved the yield of rice significantly. Hence, they served as the economically viable source, which contributed for amelioration of salt affected soils and also supported the seed germination process.

Enclosure 3

Output/Achievement of the project

Manpower trained: **1 Person (Project Fellow Ms. A. Ajilda)**

Ph.D Enrolled: **Ms. A. Ajilda (Reg. No. 0950/2014-15), Alagappa University, Karaikudi**

Publication from the project:

Strain Name	Name	Banklet No	Accession No
PBC 1	*Phormidium lucidum* ALU PBC 1	Banklet 1947432 seq1	KX780066
PBC2	*Leptolyngbya valderiana* ALU PBC 2	Banklet 1950053 seq1	KX816335
PBC3	*Plectonema terebrans* ALU PBC 3	Banklet 1953182 seq1	KX853084
PBC4	*Cyanobacterium aponinum* ALU PBC 4	Banklet 1953183 seq1	KX853085

Enclosure 4

Contribution to the society

- Conversion of salinity and alkalinity of the soil without affecting its physical nature, biological method is suitable, easiest and cost effective one.
- Cyanobacteria have been recognized as an important agent in the stabilization of soil surfaces primarily through the production of extracellular polysaccharides, which are prominent agents in the process of aggregate formation and increase in soil fertility.

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GenBank

Phormidium lucidum ALU PBC1 16S ribosomal RNA gene, partial sequence

GenBank: KX780066.1

FASTA Graphics

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  JOURNAL   Unpublished
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GenBank

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GenBank: KX816335.1

FASTA Graphics

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  TITLE     Direct Submission
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Nucleotide

GenBank

Plectonema terebrans ALU PBC3 16S ribosomal RNA gene, partial sequence

GenBank: KX853084.1

FASTA Graphics

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Nucleotide

GenBank

Cyanobacterium aponinum ALU PBC4 16S ribosomal RNA gene, partial sequence

GenBank: KX853085.1

FASTA Graphics

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  TITLE     Isolation and identification of cyanobacteria from backwater,
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