



# Production of exopolysaccharides by the cyanobacterium *Anabaena* sp. BTA992 and application as biofloculants

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## ABSTRACT

Biofloculant exopolysaccharide (EPS) production by ten (10) cyanobacterial strains comprising of five (05) heterocystous and five (05) non-heterocystous strains during their photoautotrophic growth was investigated. Highest level of EPS was produced by *Anabaena* sp. BTA992 among the investigated strains with high flocculation capacity. 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. EPS production was maximum during stationary growth phase and late phase of growth at pH 8.5. The strain was identified by 16S rRNA typing and GenBank accession number was obtained as KJ830950. The investigation revealed that this *Anabaena* sp. could be a potential candidate for the commercial EPS production and might be utilized in applications as an alternative to synthetic flocculants.

## 1. INTRODUCTION

Cyanobacteria also known as blue green algae are a group of extraordinary diverse gram negative prokaryotes that originated 3.5 billion years ago. Their diversity ranges from unicellular to multicellular, coccoid to branched filaments, nearly colourless to intensely pigmented autotrophic to heterotrophic, psychrophilic to thermophilic, acidophilic to alkylphilic, planktonic to barophilic, freshwater to marine including hypersaline [1]. Microbial polysaccharides are attracting increasing interest for their potential applications in the food, cosmetic and pharmaceutical industries, competing with other natural polysaccharides obtained from plants and macroalgae [2]. Besides the standard applications of microbial EPSs as food coatings, emulsifying and gelling agents, flocculants, hydrating agents etc., the specially anionic nature of cyanobacterial polysaccharides make them interesting for biomedical applications, since sulfated polysaccharides have been demonstrated to possess inhibitory properties against various types of viruses and tumours [3-4] and in the field of

bioremediation since they can be used to remove toxic metals from polluted waters [5]. Moreover, in recent years, interest in the exploitation of valuable EPSs has been increasing for various industrial applications and the attention towards polysaccharide producing bacteria and cyanobacteria has greatly increased. EPSs are regarded as abundant source of structurally diverse polysaccharides, some of which may possess unique properties for special applications. In the present work the effect of two main culture factors i.e. light and pH on biofloculant exopolysaccharides nitrogen fixing strain of the cyanobacterium *Anabaena* was studied.

## 2. MATERIALS AND METHODS

### 2.1. Cyanobacterial culture conditions

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 these strains were originally isolated from Indo-Burma biodiversity hotspots of north-eastern India falling under Indian territory. Cultivation was conducted in 250-ml Erlenmeyer flasks containing 100 ml of BG-11 medium [6].

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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 characterization and optimization of biofloculant exopolysaccharide production by cyanobacteria by the methods followed Tiwari *et al.* [7].

## 2.2. Exopolysaccharide assay and flocculating activity test

Two paper pins were allowed to settle at the bottom of the flasks and kept in a magnetic stirrer for 15 min for maceration, to separate the capsular slime polysaccharide for, the culture. Soluble EPS released in the medium was separated from intact cyanobacteria by centrifugation of cultures at 6600 X g at 15°C for 20 min (Centrifuge 5430 R, Eppendorf, Germany). The supernatant was concentrated to one-fourth of its original volume by evaporating in a hot-air oven (Universal Oven - 143, Narang Scientific Works, India) at 60°C for 10-12 h. The EPS in the concentrated liquid was precipitated by the gradual addition of three volumes of cold ethanol and was then kept at 4°C overnight [8]. The precipitate was washed twice by suspension in cold ethanol, followed by centrifugation. The gel-like pellet obtained after the final centrifugation was dialyzed against 5 volumes of distilled water overnight at room temperature. The dialysate was then dried at 60°C to a constant weight. Biofloculant capacity of the EPS extract was determined by a little modification of Alcian blue binding assay [9].

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.

Chemical components of the dialyzed EPS from the two strains were analysed. Total neutral sugar content was estimated by Anthrone method [10]. Total soluble protein was measured according to Hebert *et al.* [11]. Uronic acid content from the hydrolysed EPS was determined spectrophotometrically using the Carbazole method [12].

## 2.3. Strain Identification

Genomic DNA was extracted by a modified xanthogenate method [13]. PCR amplification of 16S rRNA gene sequences was performed using the universal forward primer 536F and reverse primer 1488R [14]. All PCR reactions were performed in a total volume of 50 µl containing 200 µM dNTPs, 0.3 µM of each primer, 1× Taq buffer, 5 U Taq DNA polymerase, and 2 µl of genomic DNA. Amplification was performed in a thermal cycler (Mastecycler 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). Partial 16S rRNA gene sequence was obtained and retrieved accession number from NCBI GenBank.

## 2.4. Time course assay of EPS production and flocculation at different pH

The effect of initial pH of the production medium was also assessed in the range of (5.0-10.0) using 0.1 M H<sub>2</sub>SO<sub>4</sub> and NaOH, at different growth phases. Experiments were carried out in triplicates to reproduce the experiment and standard deviations were also obtained.

## 3. RESULTS AND DISCUSSION

### 3.1. EPS characterization and flocculating activity

All 10 of the examined cyanobacterial strains were screened for EPS production by separating and recovering large molecules from the supernatant of cultures. *Anabaena* sp. BTA992 produced the highest level of exopolysaccharide (1.27±0.09 mg ml<sup>-1</sup>). The total amount of EPS includes the released polysaccharides in the medium and capsular or slime polysaccharides. The total EPS and flocculating activity of the EPS producing strains are indicated in table 1. The biochemical composition of EPS for the highest producing strain, *Anabaena* sp. BTA992 is indicated in figure 1. The strain utilized in this study produced large amounts of EPS relative to the amounts reported for other cyanobacteria, reaching 1.7 mg ml<sup>-1</sup> of culture. The values observed in the present study were among the highest reported for any EPS-producing cyanobacterium or EPS-producing lactic acid bacteria [15].

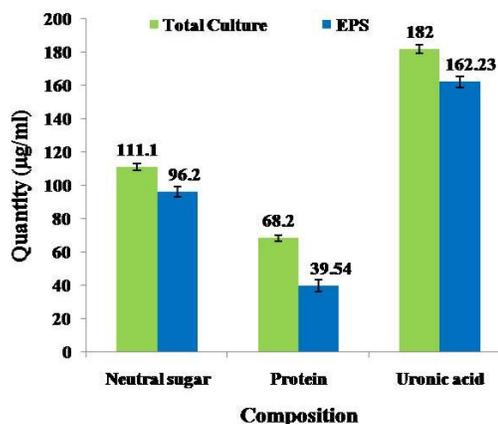
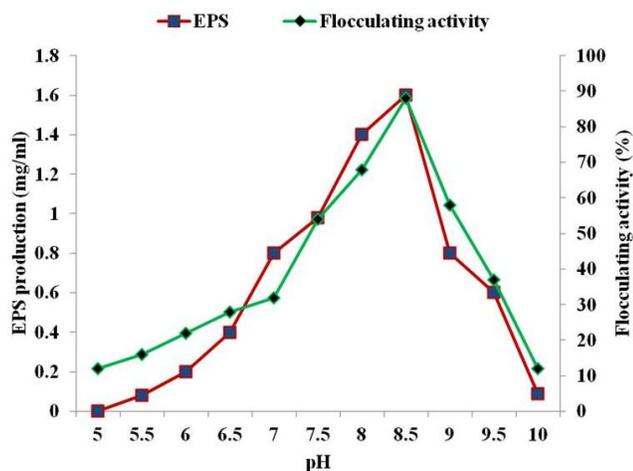


Fig. 1: Biochemical composition of EPS and total culture of *Anabaena* sp. BTA992 (with standard deviations, n =3)

A chemical analysis of the strain with highest EPS production demonstrated the presence of neutral sugars, soluble proteins and uronic acid. A diverse range of polysaccharides, with various sugars, uronic acids and proteins, have been observed previously in cyanobacterial EPS [16]. Analysis of EPS production by the unicellular cyanobacterium, *Cyanothece* sp., demonstrated a considerable quantity of EPS that contained various sugars and uronic acid [17]. The presence of uronic acids and sulfate groups confer on cyanobacterial EPS, a negative charge that contributes to efficient sequestering of cations, specifically those of heavy metals [18]. The strain produced biofloculant within the pH range (7-9) as depicted in figure 2. The optimal activity of 88% was found at

pH 8.5, after which flocculating activity declined, indicating that the isolate was slightly alkalophilic.



**Fig. 2:** Extracellular polysaccharide production and bioflocculant activity of *Anabaena* sp. BTA992 in different pH range.

It also demonstrated that, the effectiveness of the bioflocculant was at a wide range. However, the flocculant production and activity efficiency slightly reduced at highly acidic medium (pH<6) and highly alkaline medium (pH>9). Environmental conditions such as light intensity, temperature, culture medium, growth stage or nutrient availability affect the composition and quantity of EPS [19]; [20]. pH of the culture medium is important for cell growth and EPS production, perhaps relating to its influences on nutrient solubility and uptake, enzymatic activity, cell membrane morphology, byproduct formation and redox reactions [21-25], The flocculating activity of MMF1 isolated from the screening medium was 82.9% [26] which can be compared with 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<sup>-1</sup> with the maximum at 1.0 mg l<sup>-1</sup> [27].

**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	<i>Anabaena</i> sp. BTA 992	1.27±0.09	74.63%
2	<i>Anabaena circinalis</i> BTA129	1.14±0.07	72.04%
3	<i>Nostoc hatei</i> BTA12	1.02±0.05	62.03%
4	<i>Calothrix marchica</i> BTA46	1.03±0.00	52.08%
5	<i>Nostoc epilipsosporum</i> BTA685	0.86±0.02	52.03%
6	<i>Oscillatoria</i> sp. BTA239	0.92±0.05	42.03%
7	<i>Phormidium fragile</i> BTA02	0.52±0.07	16.41%
8	<i>Plectonema radiosum</i> BTA32	0.54±0.02	12.03%
9	<i>Phormidium</i> sp. BTA254	0.56±0.03	7.62%
10	<i>Phormidium tenue</i> BTA261	0.42±0.05	6.46%

#### 4. CONCLUSIONS

The flocculating efficiency and chemical properties of the compound bioflocculant produced by *Anabaena* sp. BTA992

was investigated. The bioflocculant maintained wide pH range producing flocculating activity with a maximum peak of 88% at pH 8.5. The bioflocculant was found to be a glycoprotein with the presence of uronic acid which was responsible for the flocculation mechanism. Nonetheless, the high flocculation activity observed indicates prospects towards industrial applications, and in addition, further studies on process conditions are needed for the prospect of large-scale production.

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