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Studies on Characterization of Bioflocculant Exopolysaccharide of *Azotobacter indicus* and Its Potential for Wastewater Treatment

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Abstract Partially characterized bioflocculant exopolysaccharide (EPS) produced from an *Azotobacter indicus* ATCC 9540 strain reported in our previous study was further characterized, and its flocculant potential was investigated at different pH, temperature, and cations concentrations. Flocculant activity at different concentrations of EPS in the absence of cations was reanalyzed by slight modified flocculant assay. It revealed that flocculant activity increased in a concentration-dependent manner up to a certain limit, with the maximum flocculation of 72% at 500 mgL^{-1} EPS concentration, even in the absence of cations. At the concentration of 10 mgL^{-1}, $CaCl_2$ showed more significant activity (92%) than $AlCl_3$ and $MnSO_4$. Differential scanning calorimetry study and flocculant assay revealed high temperature stability of EPS up to 97 °C. Molecular weight of the EPS determined by size exclusion chromatography was found to be approximately 2×10^6 kDa. Investigation on flocculation efficacy of the characterized EPS for wastewater treatment of dairy, woolen, starch, and sugar industry suggested it to be effective and stable at wide pH range of 5–10. Wastewater treatment with biopolymer at 500 mgL^{-1} showed reduction in biochemical oxygen demand (38–80%), chemical oxygen demand (37–79%), and suspended solids (41–68%). This study suggests that *Azotobacter* polymer has high potential in wastewater treatment as bioflocculant and can be used as a potential alternative to chemical flocculants.

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Introduction

Human activities for development of industrialization have led to increase in the discharge of waste and wastewater containing organic and inorganic pollutants. Annually large amount of wastewater, dyes are produced with lots of suspended solid material of corn, milk protein, sugar, and various solid particles during the production and processing of food, dairy, sugar, and textile like products [1, 2]. Recovering such suspended solids not only decreases the amount of pollutants for discharge but also increases the income for the factories as the recovered solids can be used as feed additives for animals. In most factories, settling separation is most common to recover suspended solid material, but the settling time is often very long and the separation efficiency is normally low. Flocculants are usually used to accelerate or improve the settling of suspended solids in various types of wastewater. Over the last decade, a variety of flocculants comprising of inorganic (polyaluminum chloride, aluminum sulfate) and organic (polyacrylamide, polyethylene amine) have found widespread applications in several industrial and wastewater treatment processes [2–6]. Although the inorganic and organic synthetic flocculants are cost-effective and strong agents, they are not easily degraded in nature and there have been concerns on their safety. Some of them, especially polyacrylamides, contain acrylamide monomers, which are both neurotoxic and strong human carcinogens [7]. Moreover, aluminum, a major component of polyaluminum chloride [8], also induces Alzheimer's disease [2]. In contrast, the naturally occurring biopolymers show weak flocculant activities yet are mostly used in food industries as they are in tune with environmental safety with no detrimental effects on fauna and flora and biodegradable characteristics [9–11]. Therefore, the flocculating substances of biological origin are expected to be useful as easy alternatives to chemical flocculants. They can be produced uniformly and reliably on large scale by microbial fermentation. Bioflocculants are a kind of biodegradable macromolecular flocculants created by microorganisms, and bioflocculation is an aggregation process in which cells, cell debris, and organic and inorganic colloids are closely bound together, mostly by extracellular biopolymers, creating the biofloc structure [12]. The bioflocculants can be applied in various processes, such as removal of microorganisms in the fermentation industry and different industrial waste treatment of textile, cosmetic, paper, leather, pharmaceutical, and food industries [4, 13–15].

In our previous studies, we reported efficacy of bacterial strain *Azotobacter indicus* ATCC 9540 for the production of exopolysaccharide (EPS) bioflocculant [16]. The preliminary characterization of the extracted polymer by different chemical tests, Fourier transform infrared spectrometry (FT-IR) spectroscopy, and thin-layer chromatography (TLC) showed the presence of uronic acids, *O*-acetyl groups, and orcinol with suggestive indication of alginate-like polymer [16]. Presence of the groups like uronic acids, *O*-acetyl, carboxyl, and hydroxyl in a chemical structure has been suggested to be a helpful characteristic of the compound for flocculation like mechanism [17]. Indeed, the polymer produced by *A. indicus* ATCC 9540 in our studies was found to have flocculant potential at different conditions, and flocculant activity was found to be cation dependent [16]. Further characterization of polymer produced by *A. indicus* ATCC 9540 will help to understand its structural features and exact chemical characteristics, which can be helpful to decide its utility in various scientific applications. Microbial polymers have been reported for flocculant potential for the treatment of industrial wastewater [4, 6, 15]. Polymers from *A.*

indicus ATCC 9540 have not been investigated for flocculant potential to treat industrial wastewater.

In the present study, we report further characterization of the polymer produced by *A. indicus* ATCC 9540 in continuation of our previous study [16]. The utility and feasibility of the produced polymer as a flocculant for treatment of dairy, starch, sugar, and textile (woolen) industry wastewater was also investigated.

Materials and Methods

Microbial Strain and Production of EPS

A. indicus ATCC 9540 was used for EPS production. Modified nitrogen-free Ashby's mannitol containing *Madhuca latifolia* flower extract as carbon source was used as fermentation medium [16].

Solubility Index

Solubility pattern of the extracted EPS was investigated by taking the known amount of the sample (10 mg) and suspending in different polar and nonpolar organic solvents such as acetone, ethanol, hexane, and petroleum ether and slightly acidic and alkaline water separately with continuous stirring.

Differential Scanning Calorimetry

The EPS was characterized for its thermal behavior by differential scanning calorimeter (DSC; Shimadzu, Model DSC-60) under N_2 flow of 80 $mL\,min^{-1}$ and at the heating rate of 20 °C/min from 20 °C to 275 °C.

Nature of Polymer

The nature of the polymer was investigated by taking the dried EPS (10 mg) in a test tube and dissolving in 10 mL of NaCl (0.05 M). To this, cetylpyridinium chloride (CPC; 10%) solution was added with simultaneous stirring until no more precipitate of polysaccharide–CPC complex was formed.

Molecular Weight Determination

The gel filtration chromatography (Sepharose gel column, Pharmacia) packed in a glass column (B_1 2×70 cm) was performed to determine the molecular weight of the EPS bioflocculant. NaCl (0.5 M) was used as an eluant, at flow rate of 0.1 $mL\,min^{-1}$. Blue dextran and β amylase (HiMedia, India) were used as molecular weight standards.

Flocculant Activity of the *A. indicus* ATCC 9540 EPS

Flocculant activity of the *A. indicus* ATCC 9540 EPS was carried out according to the method described by Kurane and Matsuyama [2] with slight modifications. In a test tube, 4.5 mL of kaolin suspension (5,000 mgL^{-1}) was added. To this mixture, 100 μL of the test bioflocculant substances was added and vortexed for 30 s and allowed to stand for 5 min at room temperature (29 °C). The absorbance of upper phase at 550 nm was measured (A). In the control experiment, 100 μL of water instead of bioflocculant was added to the suspension with rest of the conditions similar as in above experiment (B). The flocculant activity (percent) was defined and calculated [2].

$$N = \frac{B - A}{B} \times 100$$

The activity was expressed as the mean value of triplicate determination.

Effect of Cations, Temperature, and pH on Flocculant Activity

Effect of various cations on the flocculant activity of EPS was investigated by taking $MgCl_2$, $CaCl_2$, $FeCl_3$, and $AlCl_3$ as cation sources at desired concentrations (milligrams per liter) in kaolin solution. The optimum concentration of each cation required for the highest flocculation was determined. The effect of temperature and pH on flocculant activity of the EPS was determined by pre-treating polymer solution at different temperatures and range of pH buffers. Different temperature and pH conditions were also maintained during treatment assay.

Lab-Scale Studies of EPS for Industrial Waste Treatment

Wastewater from starch, sugar, dairy, and textile (woolen mill) industries were selected for these experiments. Different physicochemical parameters like pH, suspended solids, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) of each of the wastewater sample were measured before and after EPS treatment and recorded. Each wastewater sample (500 mL) was taken in a 1,000-mL beaker, and the EPS flocculant (250 mg) and $CaCl_2$ (5 mg) were then added to each beaker. The contents were blended at 200 rpm for 1 min followed by blending at 60 rpm for another 5 min at 40 °C. The treated wastewater samples were left to settle for 10 min, and the supernatant was used for further physicochemical analysis. The procedure was repeated in triplicates for wastewater samples from each source.

Results and Discussion

The EPS production by *A. indicus* ATCC 9540 was found to be consistent with our previous findings [16]. Mahua flower extract (*M. latifolia* L), a natural substrate at the concentration of 20 mgL^{-1}, gave maximum recovery of EPS. Maximum EPS production (6.10 mgL^{-1}) was found in the presence of 20 mgL^{-1} flower extract and 0.5 mgL^{-1} yeast extract containing Ashby's media with 180 rpm at 30 °C for 144 h, under controlled conditions in 2.5 L fermentor using optimized medium as found earlier [16]. Our previous study on the preliminary characterization of the extracted *A. indicus* ATCC 9540 EPS by chemical tests, FT-IR spectroscopy, and TLC showed presence of uronic acids, *O*-acetyl groups, and orcinol with suggestive indication of alginate-like polymer [16].

Solubility of EPS

The *A. indicus* EPS produced using fermentation showed precipitation with CPC, which is suggestive indication of the presence of acidic groups in its structure. The acidic groups interact with the quaternary ammonium ion (QN^+) of the CPC, resulting in the formation of polysaccharide–CPC complex. This reveals the acidic and anionic nature of polymer resembling with the report of Scott [18]. Solubility tests of EPS in various organic and inorganic solvents revealed its insolubility in range of organic solvents and solubility in slightly acidic and alkaline water. This indicates the polar nature and presence of strong hydroxyl groups in the polymer. The solubility only in aqueous indicates following the solubility principle “like dissolves like” [19]. The hydroxyl groups present within the polysaccharide have the possibility of hydrogen bonding with one or more water molecules. Thus, the polymer could imbibe water, swell, and even dissolve partially [20]. The insolubility of polymer in organic solvents may be due to the number of hydroxyl groups present in the polymer, which resulted in the building up of strong forces of attraction between polysaccharide molecules [19, 21].

Differential Scanning Calorimetry Analysis and Effect of Temperature on Flocculant Activity

The effect of temperature on EPS was carried out by DSC analysis. It revealed that the polymer has high stability at broad range of temperatures from 40 °C to 97.66 °C (Fig. 1). The melting point of EPS was found to be 97.6 °C and its degradation started above 200 °C. Besides this, the experiment of pretreated EPS at different temperatures and flocculation at different temperature in treatment revealed that the flocculant capacities increased up to 47 °C and then remained constant up to 97.66 °C and suddenly decreased above 97.66 °C (Fig. 2). As temperature tolerance of polymer plays an important role to make decision on suitability of EPS for waste treatment, our findings of DSC and flocculation at different temperature range depict the suitability of the bacterial EPS for industrial waste treatment.

Molecular Weight Determination

Molecular weight of the polymer determined by size exclusion chromatography was found to be approximately 2×10^6 kDa. Among three polymers, β amylase eluted earlier while bacterial polymer eluted with dextran. This indicates that the approximate molecular weight of polymer sample could be below β amylase and close to dextran. Although the exact

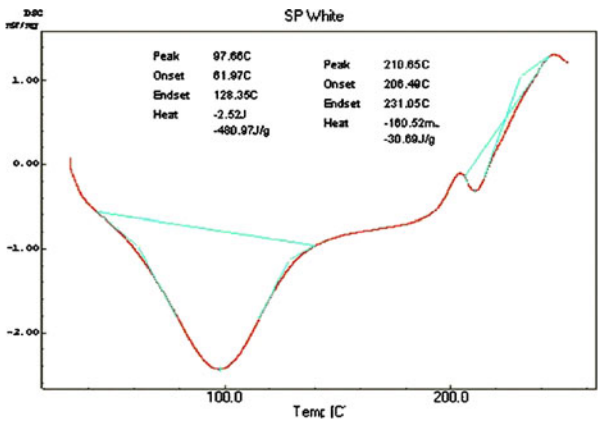
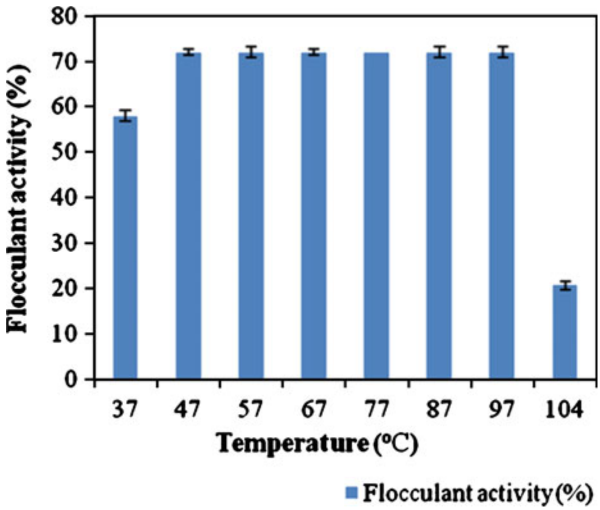


Fig. 1 DSC analysis of the exopolysaccharide

Fig. 2 Effect of temperature on flocculant activity



molecular weight is not determined due to the unavailability of marker, presence of high molecular mass of *Azotobacter* polymer was confirmed by comparing with dextran. This is a significant characteristic for exploitation of biopolymer as flocculant agent. Flocculation with high molecular weight bioflocculants involves more adsorption points, stronger bridging, and higher flocculant activity than flocculation with a low molecular weight bioflocculant [4].

Effect of pH and Cations on Flocculant Activity

Previously reported flocculant activity and potential of the polymer [16] was reanalyzed by flocculant assay of Kurane et al. [13] with slight modifications, i.e., by depriving the calcium carbonate cations. We found surprising observation that even in the absence of cations like calcium carbonate EPS showed the maximum activity 72% at 500 mgL^{-1}. To determine the exact role of cations, we used different cations at different concentrations and at range of pH. Calcium carbonate ions at the concentration of 10 mgL^{-1} were found to have momentous effect on flocculant activity. Hence, concentration of calcium ions plays a critical role in flocculant activity. Flocculant activity of polymer was less at pH 4.0 in the presence of every cation used in this study while as the pH increased, the flocculant activity also increased and attended the premier activity (92%) in the presence of 10 mgL^{-1} calcium carbonate (Fig. 3). $AlCl_3$ and $MnCl_3$ also exhibited significant increase in flocculant activity at broad pH range from 4.0 to 10.0 while $FeCl_3$ did not show any significant result (Fig. 3). Flocculant activity started ceasing above pH 10.0 while at acidic pH also activity lowered. This may be due to alkaline and acidic degradation of the polysaccharide which might be causing several changes such as molecular rearrangement of its residue or fragmentation of the polysaccharide chain [22, 23]. It seems that Ca^{2+}, Al^{3+}, and Mn^{3+} accelerate the initial adsorption of the biopolymer on kaolin particles. This synergistic effect with cations on kaolin flocculation indicates that cationic effects are a result of neutralization of zeta potential by decreasing the negative electrical charge of kaolin particles and the biopolymer flocculant [24–26]. This points out that the flocculant acts as a polyelectrolyte which aggregates the kaolin by chemical bridging to form a flocculant network. The highest effect of Ca^{2+} is one of the significant characteristics because trivalent

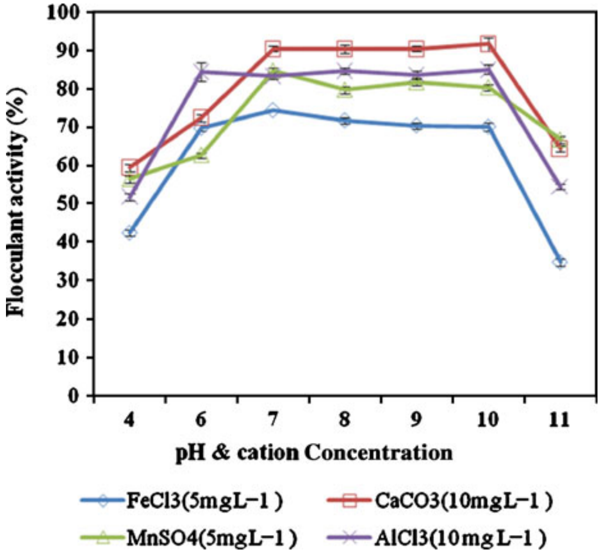


Fig. 3 Effect of pH and cations concentration on flocculant activity

cations are difficult to remove and can cause environmental problems. Fe^{3+} forms the gelatinous precipitate, which was more difficult to remove. Similarly, Al^{3+} gives rise to environmental problem; therefore, $CaCl_2$ has own coagulant aid.

Lab-Scale Studies of EPS for Industrial Waste Treatment

The physicochemical characteristics of the wastewater samples collected from different industries are presented in Table 1. Bioflocculant treated waste samples from each industry exhibited reduction in BOD and COD and suspended solids in comparison with untreated waste samples (Table 2). The highest reduction of COD and BOD was found for sugar industry waste, i.e., 76% and 80%, respectively. Bioflocculant significantly reduced suspended solids up to 63% (Table 2). These results indicate the potential of EPS as imperative flocculant agent at broad temperature and pH range for various industrial waste

Table 1 Physicochemical characteristics of the different untreated industrial wastewater samples

S.N.	Parameters	Wool industry	Starch industry	Sugar industry	Dairy industry
1	pH	9.0	5.0	7.0	7.5
2	SS (mgL^{-1})	100	6,324	450	760
3	BOD (mgL^{-1})	750	2,570	350	240
4	COD (mgL^{-1})	650	3,677	630	1,240
5	SO_4 (mgL^{-1})	910	322	30.53	22
6	Cl (mgL^{-1})	125	100	30.75	105
7	TN (mgL^{-1})	150	750	100	184
8	TC (mgL^{-1})	120	300	2,700	110

The values are means of triplicate determinations

SS suspended solids, *TN* total nitrogen, *TC* total carbon

Table 2 Waste analysis before and after EPS treatment

Industry	Parameter							
	pH		SS (mgL^{-1})		BOD (mgL^{-1})		COD (mgL^{-1})	
	C	Ta	C	Ta	C	Ta	C	Ta
Dairy	7.2	6.5 (± 0.08)	760	447.8 (± 0.52)	340	85.03 (± 0.16)	1,240	780.65 (± 0.33)
Woolen	9.0	8.0 (± 0.04)	100	55.16 (± 0.15)	750	464 (± 2.94)	650	211.66 (± 2.05)
Starch	5.2	6.2 (± 0.04)	6,324	3,508.6 (± 1.67)	2,570	1,283.5 (± 2.54)	3,677	739.33 (± 1.64)
Sugar	7.1	7.5 (± 0.08)	450	167 (± 0.57)	350	69.14 (± 0.16)	630	148.82 (± 0.41)

Values represent mean ± SD (00.00)

C before EPS treatment, *Ta* after treatment with 500 mgL^{-1} EPS at 47 °C

treatments. Extracted polymer was already reported for its high polysaccharide and low protein content (i.e., 97.7:2.3), acetyl groups, carboxyl group (–COOH), and hydroxyl (–OH) groups [16]. Presence of carboxyl and hydroxyl groups has more adsorptive forces which results in aggregation; hence, they may be the preferred groups for flocculation process [17]. This is a significant characteristic because (–COOH) groups might be used as functional groups to link this polysaccharide to starch like natural and synthetic polymers to form new polysaccharides having unique properties [27]. These results are in agreement with the report on EPS recovered from *Rhodococcus erythropolis*, *Bacillus mucilaginosus*, and heteroglycan flocculant by haloalkalophilic bacterium, *Bacillus licheniformis* [2, 4, 21, 28, 29]. High polysaccharide content is one of the noteworthy characteristics of flocculant from our studies because flocculants with high polysaccharide content are usually more heat resistant in comparison with bioflocculants with high protein content [4, 21, 30]. Bioflocculants that are not made of sugars as the main flocculant components show more sensitivity to temperature [5]. Bioflocculants with higher protein content are usually less heat stable as protein can be destroyed upon heating; hence, they may be of less importance in industrial waste treatment. Bioflocculants produced by *R. erythropolis* [13] and *Aspergillus sojae* [31] were found to be stable after heating, and their flocculant activity was more than 50% of the initial activity after heating for 15 min in boiling water [32].

In this study, the flocculant potential of the *A. indicus* EPS was evaluated using different industrial wastewaters with broad range of pH and compositions. The suspended matter immediately formed numbers of small flocks after addition EPS and $CaCl_2$. The settling velocity of particles was increased; as a consequence, the volume of solids remained was found to be 25, 30, 43, and 40 mL in starch, dairy, sugar, and woolen industry waste, respectively, of the total 500 mL of waste within 10 min. The settling activity without $CaCl_2$ treatment was observed to be comparatively slow after bioflocculant addition alone. The volume of solid remained after 30 min was found to be 150, 132, 90, and 78 mL of the total 500 mL of starch, dairy, sugar, and woolen industry waste, respectively. This proves that $CaCl_2$ plays important role in enhancement of flocculant activity.

Conclusion

The biopolymer produced by *A. indicus* ATCC 9540 strain was found to have high molecular mass, thermostability, pH receptivity, and high flocculant activity. It possesses all the characteristics of an ideal flocculant agent required for industrial waste treatments. Recovering suspended solids not only decreases the BOD and COD load of pollutants for discharge but also influences the economy with environment friendly aspect as the recovered solids can be used as feed additives for animals. Most conventional flocculants have some adverse effects on animals and the environment; their application in this field is not desirable. Because of biodegradability, harmlessness, and lack of secondary pollution, bioflocculants have gained much wider attention and research to date. *A. indicus* strain in the study is of nonpathogenic origin; therefore, EPS produced by the strain has promise to be used for the treatment of drinking water, downstream process as well as industrial waste processing because it works at wide range of pH (5.0–10). The EPS was able to significantly reduce BOD and COD above 60% in range of industrial waste and also showed average reduction in suspended solids above 35%. Therefore, this study finds significance in the utilization of *Azotobacter* exopolysaccharide as potential natural bioflocculant.

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