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Bioflocculant Exopolysaccharide Production by *Azotobacter indicus* Using Flower Extract of *Madhuca latifolia* L

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Abstract Efficacy of *Azotobacter indicus* ATCC 9540 strain for production exopolysaccharide (EPS) bioflocculant was investigated. Mahua flower extract (*Madhuca latifolia* L), a natural substrate at the concentration of 20 g L^{-1}, gave maximum recovery of EPS followed by sucrose and mannitol as compared to other carbon sources after 172 h. Yeast extract was found to be the most effective nitrogen source as compared to beef extract, sodium nitrate, ammonium sulfate, casein hydrolysate, and urea for the production of EPS. EPS production was increased in presence of nitrogen (5.51 g L^{-1}) as compared to nitrogen-free medium (3.51 g L^{-1}), and fermentation time was also reduced by 28 h. Maximum EPS production (6.10 g L^{-1}) was found in the presence of 20 g L^{-1} flower extract and 0.5 g L^{-1} yeast extract containing Ashby's media with 180 rpm at 30 °C at 144 h, under controlled conditions in 2.5 L fermenter using optimized medium. The isolated EPS showed cation-dependent flocculating activity. Concentration of EPS played an important role in bioflocculating activity which increased in a concentration-dependent manner up to a certain limit, with the maximum flocculation of 72% at 500 mg L^{-1} concentration but remained almost static after this concentration. Extracted polymer was characterized by different chemical tests, FT-IR spectroscopy, and TLC which showed presence of uronic acids, *O*-acetyl groups, and Orcinol with suggestive indication of alginate like polymer. This study suggests that use of *M. latifolia* L. flowers can be a potential alternative bioresource for production of exopolysaccharide.

Keywords Bioflocculant · *Azotobacter indicus* · Polysaccharide · EPS · Cations

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Introduction

A large number of microorganisms, including bacteria, microalgae, yeast, and fungi can produce extracellular polysaccharide polymers. These microbial exopolysaccharides are of potential interest from an economical point of view, depending on their structural properties and rheological behavior [1, 2]. *Azotobacter* spp. is free living, nitrogen fixing, obligate aerobes commonly found in tropical and temperate regions [3]. They are known to produce copious quantities of exopolysaccharides (EPS) [4], commonly manifest as large mucoid colonies when isolated from the natural soil habitat. The physiological functions of extracellular polysaccharides, produced by bacteria in nature, have been widely discussed [2]. The ability of bacteria to surround its cell in a highly hydrated EPS layer may provide it with protection against desiccation and predation by protozoan or phage attack [2]. Cells buried within a polymer matrix may be inaccessible to antimicrobial agents such as antibiotics [5] or affect the penetration of toxic metal ions [6]. They may also protect nitrogenase activity against high oxygen concentration [7], enable free-living bacteria to adhere and colonize to solid surfaces where nutrients accumulate [5], or participating interactions between plant and bacteria [8].

Many polysaccharides produced by bacteria have characteristic properties in rheology and physiological activity different from natural gum and synthetic polymers. They are biodegradable and less harmful to environment than synthetic polymers. For these reasons, some bacterial polysaccharides are produced on industrial scale and used as raw materials for food processing, medicinal, and industrial preparations [2]. Bioflocculants are the polymers produced by microorganisms during their life cycle. The organic flocculants widely applied in industrial fields are increasingly proved to be harmful to environment and dangerous source of pollution. The bioflocculants are considered as harmless agents because of their biodegradability and nontoxic nature. It has been reported that the algae, fungi, and bacteria produce flocculating agents with various properties [9–11]. There is a growing interest to find biologically based flocculants. Exopolysaccharides produced by microorganisms have good promise to act as good flocculating agents [12–14, 57].

Azotobacter indicus ATCC 9540 strain has been studied for fermentatively hydrogenating and reducing ketoisophorone to produce optically active [4R,6R]-4-hydroxy-2,6,6-trimethyl-cyclohexanone useful as an intermediate in the production of optically active carotenoids, and intermediates in the production of these carotenoids (US Patent 3988205 [15]) and effect of gibberellic acid on this strain have been investigated [16]. However, there is no report on production of an EPS bioflocculant by this strain. To develop cost-effective process for EPS production, there is need to investigate potential of microorganisms using alternate bioresources, especially cheaper carbon sources. *Madhuca latifolia* L. is a forest tree belonging to *Sapotaceae* family and abundantly available in the Indian subcontinent [17]. Its flowers are very rich in fermentable sugars which contain about 30–36 g^{-1} [17]. This paper describes the laboratory study to assess efficiency of *A. indicus* ATCC 9540 to produce bioflocculant EPS using extract of *M. latifolia* L. Biochemical and physicochemical features of EPS are also investigated.

Materials and Methods

Microbial Strain, Culture Medium, and Inoculum Preparation

A. indicus ATCC 9540 bacterial strain obtained from ATCC was grown in the culture medium of nitrogen-free Ashby's mannitol containing the following ingredients (g L^{-1}):

mannitol, 2; potassium orthophosphate, 0.22; sodium chloride, 0.2; potassium chloride, 0.1; calcium carbonate, 5. The carbon source and the salt solution (pH adjusted to 7.4 before autoclaving) were autoclaved separately. The culture medium was incubated at 30 °C for 48 h and used as inoculum, 2 ml of inoculum (5×10^8 CFU/ml) inoculated for fermentation.

Effect of Carbon, Nitrogen, and Inorganic Salts on EPS and Cell Mass Production

Ashby's medium was used in the present study keeping in view of the growth requirements. The carbon sources and their concentrations as well as other media constituents were varied and investigated to get maximum yield. In addition to these, supplements of nitrogen source, minerals, and various physical parameters were investigated to get maximum recovery of EPS. All the experiments were carried out in triplicates.

Plant Material and Preparation of Flower Extract of *M. latifolia* L

Flowers of *M. latifolia* L. were collected and dried in shade for 8 days at room temperature (28±2 °C); 100 g dry flowers were soaked in 200 ml hot distilled water (95 °C) and incubated on shaker with 220 rpm at 29 °C for 2 h. The extract filtered through muslin cloth was used as a source of sugar in medium. Reducing sugars in the flower extract were measured as per Miller [18].

Fermenter Study

EPS production was investigated in 2.5 L fermenter (Biflo III New Brunswick, USA). Silicon oil from M/S Sigma Chemicals Co, USA was added as an antifoam agent. The fermenter was inoculated with 24 h vegetative inoculums (*v*/*v*) and fermentation was carried at 28–30 °C with agitation rates varying from 100–600 rpm and aeration 0.5 v.v.m. Dissolved oxygen and temperature were maintained at 60–10% and 28–30 °C, respectively.

Recovery and Gravimetric Estimation of EPS

Recovery of the produced EPS was carried out as per Gorin and Spencer [4]. Briefly, polymer produced in the viscous culture broth was diluted with nine volume of distilled water and then centrifuged at 20,000×*g* for 10 min to remove cell mass. Two volumes of cold propanol were added to the cold supernatant. The precipitate obtained was redissolved in deionised water and 2% cetyl pyridinium chloride solution was added with stirring. After 10 h, precipitate was collected and dissolved in 0.5-N NaCl solution. Two volumes of cold propanol were then added to obtain the precipitate, which was then washed thrice with propanol. The crude polymer was dialyzed at 4 °C against deionized water, and precipitate was dried and weighed.

Cell Weight Determination

Cell weight was determined as per the method of Gorin and Spencer [4]. Briefly, bacterial cells were grown for the period of 24 to 192 h at 29 °C and in various physicochemical conditions. Culture was centrifuged at 10,000 rpm for 10 min at 22 °C. Relative centrifugation force varied according to age of the culture and supernatant

viscosity. Sedimented cells were washed thrice with 0.85% physiological saline and air-dried on filter paper and then dried at 105 °C for 3 h in an oven.

Chemical Analysis of the Polysaccharide

Chemical analysis of extracted polymer was done by following methods. *O*-acetyl content was determined by using acetyl choline hydrochloride as a standard [19]. Uronic acid content was determined by glucronic acid as a standard [20]. Biopolymer was estimated by Orcinol $FeCl_3$ [21].

Carbohydrate and Protein Content of Bioflocculant

The total carbohydrate content was measured by method of Dubois et al. [22], monitoring the absorbance at 490 nm, with glucose as a standard. The total protein content was estimated using method of Bradford [23] with bovine serum albumin as standard. The cell mass was measured gravimetrically.

Flocculating Activity of EPS

Flocculating activity of the EPS was measured by turbidity of suspension of kaolin clay as per Kurane et al. [9]. In a test tube, 4.5 ml of kaolin suspension (5,000 mg L^{-1}) was added and mixed with 0.25 ml of $CaCl_2$ solution (90 mM). To this mixture, 100 μl of the test bioflocculating substances were 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 flocculating activity (%) was defined and calculated as $[(B-A)/B]\times 100$. The activity was expressed as the mean value from triplicate determination [9].

Hydrolysis of EPS for Monomer Analysis

For monomer analysis, purified polysaccharide and standard alginate were separately hydrolyzed with 2 M trifluroacetic acid, at 104 °C for 3 h. Hydrolysate was diluted by distilled water and desalinized on a column (4×0.5 cm) of Dowex-50× (Sigma Chemical Co., USA). The monomers were eluted by 20 ml distilled water and concentrated at 50 °C under reduced pressure by using vacuum evaporator (Buchi, B-480, Switzerland). Eluted samples were adjusted to pH 7.15 with 0.1 N NaOH and thin layer chromatography (TLC) analysis of the spots was performed on standard silica gel 60 F_{254} plates (E. Merck, Germany), with ethyl acetate/acetic acid/water (9:2:2) as solvent system, and the spots were detected by spraying with P-anisidine-HCl reagent followed by heating at 120 °C for 5 min. Authentic monomers of mannuronic, guluronic acids, and alginate standard were treated under same conditions of hydrolysis and were run along with the test samples.

FT-IR Spectroscopy of EPS

The EPS was characterized using Fourier transform infrared spectrophotometer (Testscan Shimadzu FTIR 8000, Japan). The dried polymer was ground with KBr powder and pressed into pellet for FTIR spectroscopy, frequency range 4,000–450 cm^{-1}.

Results and Discussion

Different media have been reported for culturing *Azotobacter* spp. [4, 24]. The basic components of various media for nitrogen fixers are almost similar with variation in the type of carbon source (sugar), its concentration, and growth conditions. In these studies, we investigated potential of *M. latifolia* L flower extract as a carbon source. Typically, 100 ml extract of 60 g of flowers contained 20% fermentable sugars. Various concentrations of these fermentable sugars were tested for EPS production.

Effect of Aeration, Temperature, and pH on Bioflocculant EPS Production

Various shaking speeds were used to study the influence of aeration on EPS and biomass production. The bacteria were inoculated in nitrogen-free Ashby's mannitol medium, and flasks were incubated on different shaking speeds of 100 to 200 rpm. EPS and biomass were measured gravimetrically at the interval of 24 h up to 196 h at 30 °C. Increase in EPS and biomass production was found with increasing aeration with maximum production at 180 rpm after 172 h suggesting a key role of shaking speed in EPS production. Alginate production depends on the dissolved oxygen tension (DOT) of the culture broth, which shows initial increase followed by plateau at 1.5% (DOT) and then decrease at higher DOT values [25–27]. Increase in DOT of culture broth results in increase in respiration of microorganisms [26]. Therefore, the rate of bacterial EPS production decreases at high DOT because most of the carbon source is burnt as carbon dioxide. Oxygen limitation has proven to be disadvantageous for bacterial EPS production. Under low DOT, abundant poly-hydroxybutyrate (PHB) was accumulated in cells of *Azotobacter vinelandii* up to 32% of dry cell weight [27]. The result obtained in these studies could provide useful information for EPS production by *A. indicus* strains. The effect of temperature on biomass and EPS production was investigated at cultivation temperature (25, 30, 35, 40 °C) in incubator shaker at the interval of 24 to 196 h. The highest biomass and EPS production were obtained when bacteria were cultivated at 30 °C for 172 h. Previous reports have shown bioflocculant production by microbes during log phase [10, 28] while our studies bioflocculant production started after 48 h and maximum production was found at stationary phase (Fig. 8). The biomass and EPS production was increased with increase in temperature up to 30 °C but decreased after 35 °C. The organism showed growth and EPS production in wide range of pH with the maximum being at the pH 7.0. Sugar uptake depends on external pH; therefore, adequate control of pH value is essential in both batch and continuous fermentation of EPS production [2].

Effect of Inorganic Salts on Bioflocculant EPS Production

EPS production was increased with increase in $CaCO_3$ concentration and remained almost constant from 200 to 1,000 mg L^{-1} but decreased beyond the concentration of 1,500 mg L^{-1}. Concentration of $CaCO_3$ at 1,000 mg L^{-1} enhanced the recovery of EPS, but its concentration beyond 2,000 mg L^{-1} was found to be inhibitory for EPS production (Fig. 1). Variation of NaCl and KCl did not affect EPS and cell mass production but concentration beyond 2 g L^{-1} showed inhibitory effect (data not shown). Phosphate (KH_2PO_4) at 500 mg L^{-1} concentration showed maximum EPS production, but further increase in phosphate reduced EPS production (Fig. 2). EPS production was almost constant at the 10–100 mg L^{-1} concentrations of $FeSO_4$ and slightly decreased beyond 100 mg L^{-1} (Fig. 3). $MgSO_4$ at 100–500 mg L^{-1} concentration showed increase in EPS recovery, while above 500 mg L^{-1}

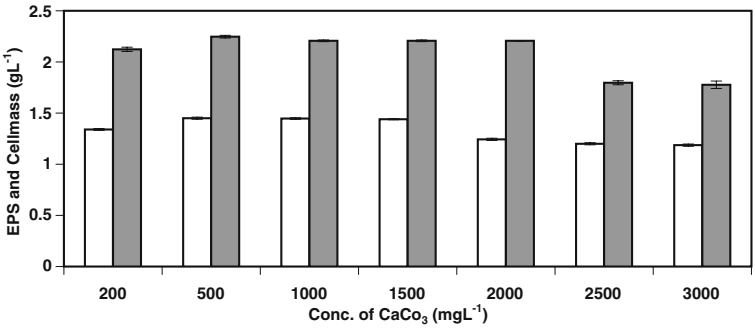

Fig. 1 Effect of calcium carbonate on bioflocculant EPS production; *unfilled bars* EPS, *filled bars* cell mass

concentration EPS production was decreased (Fig. 4). Inorganic salt such as calcium carbonate, magnesium sulfate, and potassium phosphate, play important role in physiology and growth of organism. Calcium at specific concentration stimulates the sugar uptake by decreasing Km of sugar transport, whereas at high sugar concentration, it inhibits this reaction [29]. Phosphate at 0.5 g L^{-1} concentration showed high EPS recovery in our studies. This is in agreement with that of Horan et al. [27]. EPS production was found to be increased with addition of $FeSO_4$ and $MgSO_4$, which have been reported to play critical role in EPS production by activating certain enzymes of EPS synthesis [30].

Effect of Carbon Sources on Bioflocculating EPS and Biomass Production

M. latifolia L. flower extract at 20 g L^{-1} gave maximum recovery of EPS (3.95 g L^{-1}) and cell mass (2.19 g L^{-1}), followed by sucrose and mannitol as compared to other carbon sources after 172 h (Fig. 5). Sucrose and mannitol (20 g L^{-1}) were also quite effective in EPS and biomass production with 3.76, 1.52, 2.19, and 2.20 g L^{-1}, respectively at 172 h (Fig. 5). On the other hand, yields were less than 50% with use of glucose, fructose, and maltose while EPS was not produced when starch was used as a carbon source (Fig. 5). *A. indicus* showed high EPS and cell mass in the media containing *M. latifolia* L. flower extract with 20 g L^{-1}, but EPS yield and cell mass was minimum when flower extract was used at lower concentrations as well as beyond 25 g L^{-1} in the medium (Fig. 6). The *M. latifolia* L. flower extract mainly contains sucrose as a principle component of the sugars, and interestingly, sucrose as a carbon source was more preferred by *A. indicus* showing

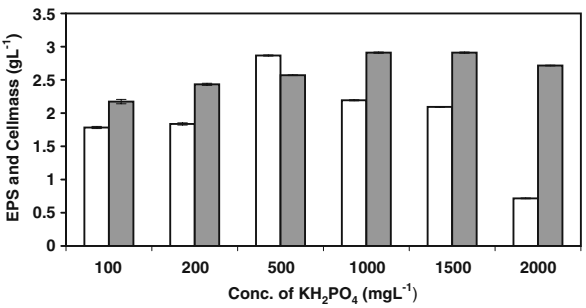

Fig. 2 Effect of phosphate on bioflocculant EPS production; *unfilled bars* EPS, *filled bars* cell mass

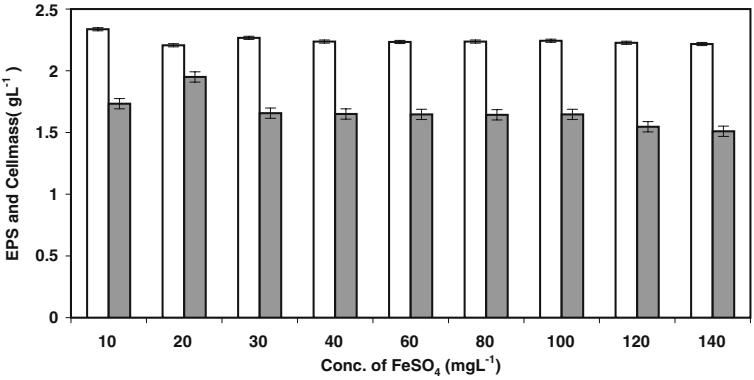

Fig. 3 Effect of iron on bioflocculant EPS production; *unfilled bars* EPS, *filled bars* cell mass

highest EPS synthesis. EPS production by *A. vinelandii* was reported to increase when 2% sucrose was used as carbon source [31]. Similar trend of increase of alginate production by *A. vinelandii* and polygalacturonic acid bioflocculant by *Corynebacterium glutamicum* with sucrose as a carbon source was reported by other researchers [13, 32, 33].

Effect of Nitrogen Sources on Bioflocculant EPS Production

Although *Azotobacteriaceae* in general and *A. indicus* are nitrogen fixers, nitrogen was supplied in the medium in the form of $NaNO_3$, $(NH_4)_2SO_4$, amino acids, yeast extract, and casein, at various concentrations to decrease the lag phase and generation time and thus period of fermentation. After optimizing carbon and other physical parameters, effect of different concentrations of various nitrogen sources from 0.1–1.0 g L^{-1} were investigated on EPS and biomass production at the interval of 24–192 h.

Yeast extract was found to be the most effective nitrogen source as compared to beef extract, sodium nitrate, ammonium sulfate, casein hydrolysate, and urea in the production of EPS and biomass (Fig. 7). The highest biomass (2.55 g L^{-1}) and EPS (5.51 g L^{-1}) were produced in the medium containing 20 g L^{-1} sucrose and 0.5 g L^{-1} yeast extract with 180 rpm shaker speed after 144 h (Fig. 7). Further increase in concentrations of yeast

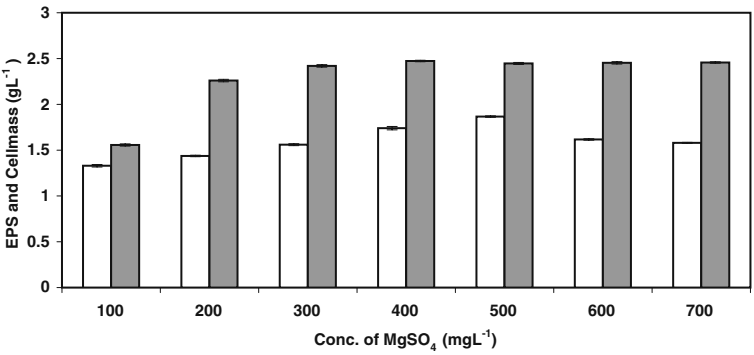

Fig. 4 Effect of magnesium sulphate on bioflocculant EPS production; *unfilled bars* EPS, *filled bars* cell mass

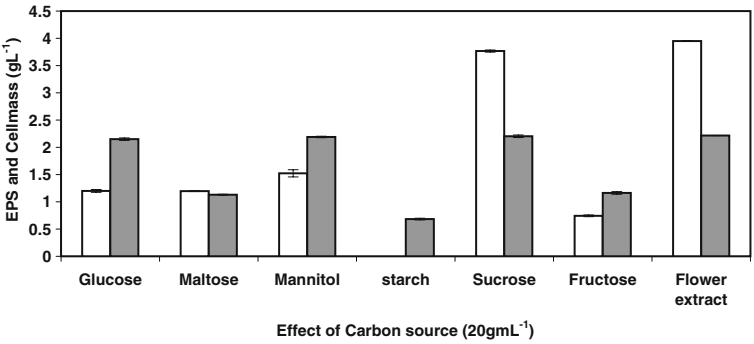

Fig. 5 Effect of carbon sources on bioflocculant EPS production; *unfilled bars* EPS, *filled bars* cell mass

extract did not alter the growth pattern and EPS production (Fig. 7). EPS production was more in the presence of nitrogen (5.51 g L^{-1}) than in nitrogen-free medium (3.51 g L^{-1}), and fermentation time also reduced by 28 h. EPS production by *A. vinelandii* found to be more when fixed nitrogen was supplied than under nitrogen-free condition [32, 34]. Similar trend for alginate production was reported by using C-14 mutant for *A. vinelandii* and Bioflocculant from *Klebsiella* sp.MYC [35, 36]. Yeast extract was found to be the best nitrogen source, possibly by a complement of vitamins and minerals besides being a complement protein. At high concentration of yeast extract, both EPS and cell mass get decreased because *Azotobacter* spp. is osmotically sensitive strain showing impaired growth and partial lysis with leakage of culture fluid. Peptone and yeast extract are not commonly added to media of *Azotobacter* sp. as they usually cause pleomorphism and make fragile cell wall strength [3, 37–39, 56]. Glycine is a principle constituent of peptone, and yeast extract causes interference with peptiodoglycan synthesis and results in the accumulation of UDP-glycopeptide precursor in cytoplasm and make osmotically fragile cells [3, 39]. Similar effects were demonstrated in case of peptone supplemented in fermentation medium of *A. vinelandii* for PHB synthesis [40]. During fermentation, EPS production started after 72 h and was highest during 144–192 h, but after that, there was no appreciable increase in EPS production similar to that observed by Horan et al. [27] for alginate production by *A. vinelandii*.

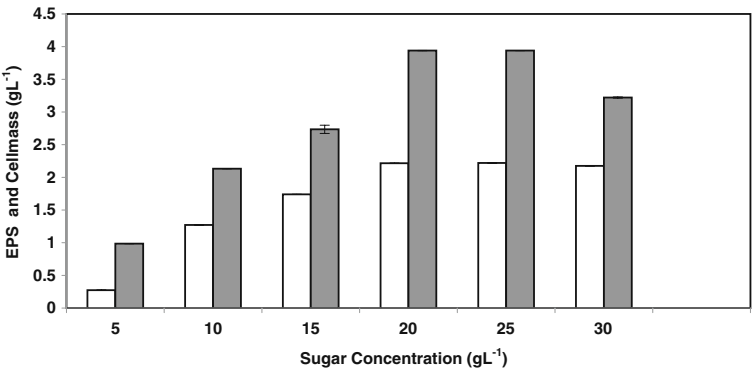

Fig. 6 Effect of sugar content of flower extract on bioflocculant EPS production; *unfilled bars* EPS, *filled bars* cell mass

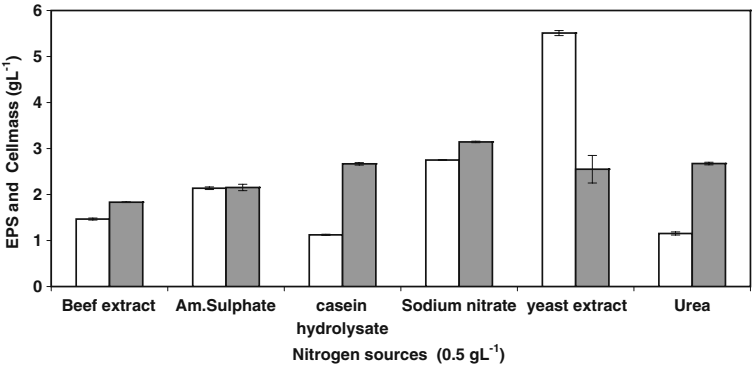

Fig. 7 Effect of nitrogen sources on bioflocculant EPS production; *unfilled bars* EPS, *filled bars* cell mass

Study in Controlled Fermenter

After optimization study in shake flasks, production of EPS was studied under controlled conditions in 2.5-L fermenter using optimized medium. Several fermentation batches were run to optimize different parameters such as agitation, aeration, dissolved oxygen and inoculum size, etc. Fermentation was carried out at 0.5 v.v.m and the agitation between 100 and 400 rpm. Agitation of 200–250 rpm was found to be most suitable for growth and EPS production. In most of runs, pH of the fermentation was not controlled but was monitored. Initial pH, after inoculation, was around 6.8 to 7.0, which decreases to 4.5–4.0 at the end of the fermentation. In some batches, pH was controlled at 7.0, but it did not result in improvement in the EPS production as compared to the fermentation run without pH control.

A typical fermentation profile in terms of DO, pH, and EPS is shown in Fig. 8. Maximum EPS production (6.10 g L^{-1}) was found in presence of 20 g L^{-1} flower extract and 0.5 g L^{-1} yeast extract containing Ashby's media at 144 h, with 180 rpm at 30 °C. Original pH dropped to 4.2 after 144 h (Fig. 8). This is a typical feature of *Azotobacter* spp. and has been associated with slime production [41]. The decrease in pH is thought to be due to oxidation of glucose to gluconic acid [31]. Although, there are few reports on bioflocculant production using industrial wastes like fermenting liquors and oil field waste [36, 42]; as per our knowledge, this may be the first report on bioflocculant production

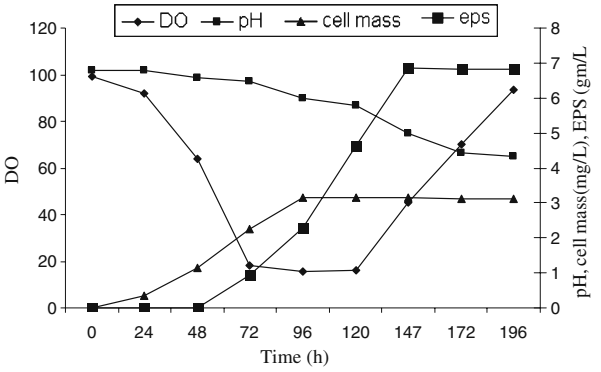

Fig. 8 Fermentation profile of bioflocculant EPS

using herbal source, i.e., flower extract of *M. latifolia* L. In addition to this, the flocculant EPS produced in this study is also quite higher than the previously reported production on industrial wastes [43, 44].

Effect of EPS Concentrations on Bioflocculating Activity

Concentration of EPS played an important role in bioflocculating activity which increased in a concentration-dependent manner up to a certain limit, with the maximum flocculation of 72% at 500 mg L^{-1} concentration but remained almost static after this concentration (Fig. 9). Further increase in concentration did not increase the flocculating activity (Fig. 9). Various microorganisms, like *Bacillus polymixa*, *Rhodococcus erythropolis*, *Nocardia amarae*, and *Streptomyces griseus*, producing different types of polysaccharides are reported to exhibit flocculation activity [11, 12, 14, 45]. Although, the previously reported flocculant activity (78–99.5%) is higher than present report, the amount of polymer required for flocculation is also quite higher than present investigation, i.e., 700 mg l^{-1} and above [10, 46, 47]. *Azotobacter* spp. are well known for its nitrogen-fixing and EPS-producing capacity [3, 4, 31], but as per our knowledge, *Azotobacter* strain was not reported earlier for its bioflocculant producing activity.

Chemical Composition

Extracted polymer was characterized by different chemical tests. Polymer was found to contain acetyl groups, which is a distinguishing character of bacterial alginate; the uronic acid content was 4.84%. All these and positive Orcinol–$FeCl_3$ tests are indicative of presence of alginate like polymer. The FT-IR spectrum of purified polymer showed a broad intense absorption band at 3,343 cm^{-1} and a sharp band at 1,651 cm^{-1} which suggests the presence of carboxyl group (—COOH) and hydroxyl (—OH) groups, respectively. This was also supported by the absorption bands at 1,377 cm^{-1} for C—O and O—H deformation. Furthermore, the additional band at 1,071 cm^{-1} (cyclic C—O) indicates the presence of guluronic acid, mannuronic acid and *O*-acetyl ester (Fig. 10). FT-IR spectrum of EPS of the present study showed presence of carboxyl and hydroxyl groups (Fig. 10), which are the preferred groups for flocculation process, similar to that reported in polyelectrolytes [48], in agreement with the report on EPS recovered from *R. erythropolis, B mucilaginous*, and heteroglycan flocculant by *Haloalkalophilic* bacterium [11, 43, 47, 49]. Different bacteria can produce bioflocculants with various chemical compositions.

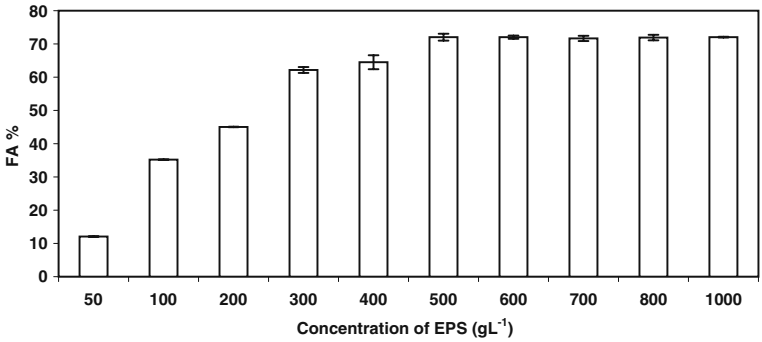

Fig. 9 Effect of EPS concentration on flocculant activity; *unfilled bars* EPS, *FA* Flocculant activity

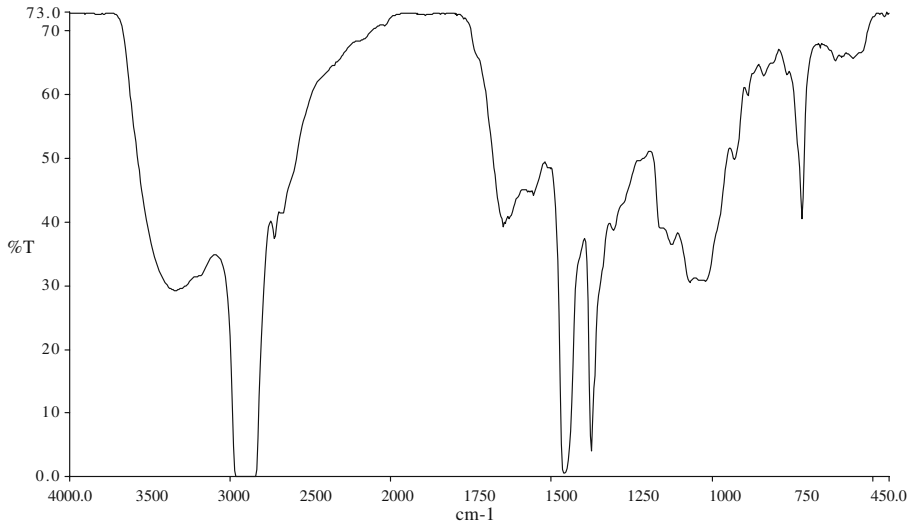

Fig. 10 IR spectroscopy of EPS bioflocculant

Deng et al. [47] reported production of bioflocculant composed of carbohydrates and proteins (at the ratio 76.3:21%), while other researchers have reported production of bioflocculants with constituents like proteins, glucosamine, and galacturonic acid [49, 50].

Chemical analysis of the polymer revealed that production of total sugar to protein content was 94.3 to 5.7 *w*/*w*. This is in agreement with previous report on bioflocculant by *Haloalkophilic bacillus* [43]. High polysaccharide content is one of the noteworthy characters of flocculant from our studies because high polysaccharide containing flocculants are usually more heat-resistant in comparison to protein bioflocculants [43, 47, 51]. TLC performed for hydrolysate of purified polymer on standard silica gel 60 F_{254} plates with ethyl acetate/acetic acid/water (9:2:2) as solvent system, P-anisidine-HCl spraying reagent, and heat treatment at 120 °C for 5 min showed two distinctly separated spots on the TLC plates. The Rf values of two separated spots were exactly matched with Rf values of standard monomer viz. guluronic acid (Rf 0.82), mannuronic acid (Rf 0.66) as well as to the hydrolysates of standard alginate. EPS of specific composition with respect to uronic acids and *O*-acetyl content are produced when sucrose is used as a carbon source. This is an important characteristic of *Azotobacter* spp. alginate. Alginate is a hetero-polysaccharide made up of mannuronic, guluronic acids, and *O*-acetyl groups. Alginate is a well-known polymer produced by *Azotobactereaceae* family in different chemically defined medium [4, 52], although other polymers by *Azotobacter* spp. have been reported [53]. The results of the polymer characterization from this study showed *O*-acetyl content (4–57%) to be similar with previously reported data [2]. However, limitation of N, C, and O was reported to affect both the conversion of carbon source into EPS and the composition of EPS in *P. mendocina* fermentation [54].

Conclusion

This study suggests that use of *M. latifolia L.* (Mahua flowers) flowers can be a potential alternative bioresource for production of exopolysaccharide apart from cane sugar and

conventional carbon sources. Recently, Shamala [55] investigated the use of *M. latifolia* L flower extract as sugar source for polyhydroxy alconate production by microorganisms *Spingomonas* sp., *Bacillus-256*, and *Rhizobium melilotii*. In the present study, *A. indicus* was found to produce capacious quantities of exopolysaccharide bioflocculant with *M. latifolia* L flower extract as a carbon source. The utilization of *M. latifolia* L. flowers as a natural substrate seems to be of an additional advantage to *A. indicus* wherein it can use organic materials along with sugars to synthesize polymer. In this study, bioflocculant EPS was produced by *A. indicus* for the first time, and conditions for its production were optimized. These biologically based flocculants may facilitate application in food, beer, environment management, and agro-industries. In view of above results, the Ashby's medium was modified for maximum recovery of bioflocculating exopolysaccharides as follows (g L^{-1}): flower extract, 20.0; yeast extract, 0.5; potassium orthophosphate, 0.20; $FeSO_4$, 0.04; calcium carbonate, 1; $MgSO_4$, 0.5; pH adjusted to 7.0. Fermentation carried out at 29 °C at 172 h resulted in the maximum yield. Under the optimized conditions, *A. indicus* can convert 30.50% of the supplied carbon source provided in the form of sugars in flower extract to EPS within 144 h. This trend seems to be similar to the observations during alginate production using *A. vinelandii* NCIB 9068 in Burk's medium containing sucrose as carbon source by Chen et al. [35] and Vermani et al. [53] who reported 16.50% conversion of sucrose into EPS by *A. vinelandii*. Therefore, this study finds significance in the utilization of alternative bioresources for production of exopolysaccharide bioflocculants and to understand biology and usefulness of *A. indicus* strains.

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