Production, characterization and structural modification of exopolysaccharide-based bioflocculant by *Rhizobium radiobacter* SZ4S7S14 and media optimization

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Abstract Synthesis of the exopolysaccharide-based bioflocculant *Rhizobium radiobacter* SZ4S7S14 was researched and medium optimized for enhanced production of bioflocculant. D-Mannose and yeast extract were found to be best carbon and nitrogen sources for maximal yield of bioflocculant. The bioflocculant samples obtained in different media with different carbon and nitrogen sources were further analyzed by SEM-EDX and FT-IR. FT-IR spectroscopy of the bioflocculant samples, obtained in different carbon/nitrogen sources showed slight modification of the structures of biopolymers. SEM micrographs revealed that exopolysaccharide-based bioflocculant of *R. radiobacter* SZ4S7S14 looks like bricks, and chemical structure of it can be varied due to utilized carbon and nitrogen source.

Keywords *Rhizobium radiobacter* · Exopolysaccharide-based bioflocculant · Synthesis · Optimization

Introduction

The polysaccharide-based bioflocculants are natural macromolecules or biopolymers produced by a large group of microorganisms, have been widely used in different fields (Lian et al. 2008; Zheng et al. 2008; Li et al. 2009, 2013; Wan et al. 2013; Aljuboory et al. 2013, 2014; Nwodo et al. 2014). The polysaccharide macromolecules in bioflocculants contain monomeric units such as glucose, mannose, fructose, or rhamnose, etc. (Kanmani and Lim 2013), rich in hydroxyl groups. These groups allow the control of shape, size and particle dispersion. Monomers type and molar ratio, other constituents in bioflocculants are varied due to the species and genus of microorganisms. Bioflocculants are able to flocculate solid and colloidal particles, the microbial cells of different sizes in a liquid suspension (Xiong et al. 2010). In aqueous solutions, the bioflocculants readily form different types of liquid crystals and bioflocculant-mediated processes are cost-effective, ecologically friendly (Manivasagan et al. 2015). In recent years many studies have been reported for the synthesis of bioflocculants, such as *Azotobacter*, *Bradyrhizobium* (Rasulov et al. 2016a, b), *Rhizobium* (Wang et al. 2011), *Bacillus* (Zheng et al. 2008; Lian et al. 2008; Li et al. 2009), *Streptomyces* and *Cellulomonas* (Nwodo et al. 2014), *Paenibacillus* (Li et al. 2013), *Citrobacter* (Ike et al. 2000), *Serratia* (Gong et al. 2008), *Klebsiella* (Yang et al. 2012) and *Aspergillus* (Aljuboory et al. 2013, 2014).

The current research was aimed to research the polysaccharide-based bioflocculant synthesis by *R. radiobacter* SZ4S7S14. Liu et al. (2016) recently reported some biological properties of the strain. In this survey, it was aimed to medium optimization for enhanced production of polysaccharide-based bioflocculant, and its physical–chemical characterization and possible structural

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modification. Besides, carbon and nitrogen source impact on the chemical structure of the bioflocculant was also a goal of the research.

Materials and methods

Molecular identification of the bacterial strain

The extraction of genomic DNA from bacterial cells and PCR amplification of the 16S rRNA gene were done as described by (Li et al. 2007). The extracted DNA was dissolved in 20 L TE buffer and used as the template for the PCR reactions. PCR amplifications were performed by using universal primers designed by (Wang et al. 2006). Moreover, the partial 16S rRNA sequences carried out in the present study were compared with the Genbank database via BLAST by EzBio Cloud server (<http://www.eztaxon.org>; (Chun et al. 2007).

Cultivation of *R. radiobacter* SZ4S7S14

Cultivation of *R. radiobacter* SZ4S7S14 was carried in the ISP2 medium containing (g/L): yeast extract-4; glucose-4; malt extract-10; water-1 L; pH of the medium adjusted to 7.3 and sterilized at 121 °C for 20 min (Shirling and Gottlieb 1966). For a production of bioflocculant, each flask was inoculated with 4% (v/v) of the seed culture and incubated in a shaker at 30 °C and 150 rpm for 3 days.

Production of bioflocculant

10 mL of the *R. radiobacter* SZ4S7S14 inoculum was inoculated into a 250-mL flask containing 100 mL ISP2 medium and incubated for 72 h in a shaker at 30 °C and 150 rpm. Then the fermentation broth was centrifuged at 10,000 rpm at 4 °C for 20 min. The cell-free culture supernatant, which was containing the bioflocculant, was thick liquid and also used for evaluation of flocculating activity (Yang et al. 2012).

Screening of carbon, nitrogen and cation sources for bioflocculant production was performed as described elsewhere with slight modification (Nwodo et al. 2014). D-glucose, D-fructose, sucrose, maltose and D-mannose were used as carbon sources, whereas yeast extract, peptone, tryptone, malt extract, beef extract, casamino acid were used as nitrogen sources. Salts such as KCl, NaCl, MgCl₂, CaCl₂, MnCl₂·4H₂O, FeCl₂, FeCl₃, AlCl₃ and Na₂MoO₄ were used as source of respective cations.

Isolation and purification of the bioflocculant

Bioflocculant purification was carried out as documented elsewhere (Manivasagan et al. 2015; Rasulov et al. 2016a, b). After 72 h incubation in a shaker, the culture broth was centrifuged at 10,000 rpm at 4 °C for 20 min. Two volumes of cold absolute ethanol were added to the supernatant and white cotton-like flocks were precipitated. The ethanol and the cell-free supernatant mixture were centrifuged at 10,000 rpm for 15 min and the precipitate redissolved in distilled water at ratio 1:4 (v/v). The procedure repeated twice and the purified bioflocculant was vacuum-dried to obtain a purified bioflocculant.

Determination of the flocculating activity

The flocculating rate was used as a measurement of the flocculating activity of the bioflocculant. A 0.5 g kaolin clay (average diameter 4 mm) was suspended in 100 mL distilled water, and then 5 mL 1% (wt%) CaCl₂ and 2 mL of bioflocculant (culture broth) were added to the kaolin suspension. The mixture was stirred with rapid mixing at 200 rpm, followed by slow mixing at 50 rpm and then kept still for 10 min. The absorbance of the supernatant and the blank without bioflocculant was measured at 550 nm (OD₅₅₀ and OD_{blank}, respectively) with a spectrophotometer. The flocculating rate was defined and calculated as follows:

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

where A and B are optical density (OD) of the control and sample, respectively, at 550 nm (Yang et al. 2012).

Bioflocculant composition analysis

The total sugar content of the bioflocculant was determined by the phenol-sulfuric acid method using glucose as a standard solution (Chaplin and Kennedy 1994). The protein content was measured by the Lowry-Folin method using a UV spectrophotometer (Shimadzu, Japan).

Characterization of bioflocculant

SEM-EDX (Scanning Electron Microscope-Energy dispersive X-ray) analysis of the bioflocculant was performed using a Hitachi apparatus, Japan. The FT-IR spectrum of the bioflocculant was analyzed using FT-IR spectroscopy (JASCO FT-IR 460, Daejeon, South Korea) operated at a resolution of 4 cm⁻¹. For the measurement of FT-IR spectrum, the dried sample was powdered by grinding with KBr pellets and pressed into a mold. The spectrum was

recorded at a frequency range of 500–4000 cm^{-1} (Rasulov et al. 2016; Kanmani and Lim 2013).

Results

Identification of the bacterial strain

The similarity of the strain SZ4S7S14 to *R. radiobacter* 19358(T) (AJ389904) was 100%. The phylogenetic analysis based on 16S rRNA gene sequences revealed that the endogenous strain SZ4S7S14 was identified as *R. radiobacter* (Fig. 1).

Growth profile of the strain and biofloculant production

Biofloculant production by the strain proportionally correlated with cell growth, cultivation time and flocculating activity during the logarithm phase. The biofloculant production maximum reached after 72 h of incubation and in the medium where glucose/yeast extract used as C/N sources, it was produced 3.6 g/L the polysaccharide-based biofloculant (Fig. 2). In the cultivation period, the flocculating activity also reached its maximum—92%. In the first and second days (after 24 and 48 h) of incubation a sharp drop in pH from 7.3 to

Fig. 2 Biofloculant production, flocculation activity and cultivation time correlation

5.2 was observed, which can be explained with intensive synthesis of organic acids from glucose or the presence of organic acids (uronic acids, see “[Partial chemical characterization of the polysaccharide-based biofloculant and its modification](#)”) of the biofloculant (Xiong et al. 2010; Dermilim et al. 1999). Between 60 and 72 h of incubation, pH began to increase and stabilized at about 5.0–5.1 (Fig. 3). pH change in such a manner was observed in all carbon and carbon sources tested.

Fig. 1 Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences of the strain SZ4S7S14

Fig. 3 Cultivation time, cell dry weight and pH correlation

Partial chemical characterization of the polysaccharide-based bioflocculant and its modification

Characterization of bioflocculant

SEM micrographs of bioflocculant showed that it looks like bricks with an average size of 100–350 μm (Fig. 4). Elemental analysis of bioflocculant by EDX revealed that it consists of 51.47% O and 45.79% C (Fig. 5). The polysaccharide-based bioflocculant further analyzed by FT-IR to evaluate functional groups and chemical bonds (Fig. 6). The broadest and strongest peak was observed at 3447 cm^{-1} , due to a stretching vibration of hydroxyl groups (O–H). A weak peak at 2922 cm^{-1} , known to be typical of carbohydrates, indicated C–H asymmetrical stretching vibration (Xiong et al. 2010). A peak at 1730 cm^{-1} was due to C=O. An asymmetrical stretching

Fig. 4 SEM micrograph of bioflocculant of *R. radiobacter* SZ4S7S14

peak at $1630\text{--}1670\text{ cm}^{-1}$ was characteristic of C=O stretching vibration in --NHCOCH_3 (Xiong et al. 2010). A peak at 1465.7 cm^{-1} corresponds to C=C and the broad peak at 1074 cm^{-1} corresponds to the C–O–C stretching from the glycosidic linkages and O–H bending from saccharide derivatives (Rasulov et al. 2016a).

Primary analysis showed that in the modified ISP2 medium, the strain synthesized the bioflocculant, which consists of 76.2% total sugars, 12.7% proteins and 6.53% uronic acid. 4.57% constituent of the bioflocculant remained unidentified solids.

Structural modification of the polysaccharide-based bioflocculant

FT-IR observation revealed some structural alterations in the bioflocculant, obtained after cultivation of *R. radiobacter* SZ4S7S14 in different carbon and nitrogen sources. FT-IR of 18 samples of the bioflocculant, synthesized in different C/N sources, exhibited 7 differing spectra (Fig. 7). These bioflocculant samples were produced after cultivation of *R. radiobacter* SZ4S7S14 in (as carbon/nitrogen sources) (glucose/malt extract, sample 1), (sucrose/malt extract, sample 3), (sucrose/peptone, sample 9), (mannose/casamino acid, sample 11), (mannose/yeast extract, sample 14), (sucrose/yeast extract, sample 15) and (glucose/beef extract, sample 18). An intense absorption peak, characteristic of a hydroxyl group, which can be caused by the vibration of –OH or –NH in the sugar ring, appeared at 3412.66, 3421.10, 3431.17, 3447.39 and 3447.67 cm^{-1} in the samples. The peak, which indicates C–H asymmetrical stretching vibration, typical to carbohydrates shifted and appeared in the regions from 2922.2 to 2927.0 cm^{-1} . An asymmetrical stretching peak for C=O stretching vibration in --NHCOCH_3 was observed at 1617.94, 1629.09, 1636.42, 1636.91, 1647.35, 1647.78, 1653.71 and 1654.15 cm^{-1} . And peak, corresponding to the C–O–C stretching from the glycosidic linkages and O–H bending from saccharide derivatives, was observed at about from 1074 to 1076.16 cm^{-1} . This evidence confirms that carbon and nitrogen sources not only impact on bioflocculant production but also cause changes in the bioflocculant's chemical structure.

Carbon and nitrogen sources impact on bioflocculant production and flocculation rate

Among the tested carbon and nitrogen sources, D-mannose and yeast extract contributed to high yield of bioflocculant production by the strain. Analysis showed that in the modified ISP2 medium, where D-mannose used as carbon sources, the strain produced 5.41 g/L bioflocculant (Table 1). And it was 1.15–2 times higher than when other

Fig. 5 EDX of bioflocculant of *R. radiobacter* SZ4S7S14

Fig. 6 FT-IR of bioflocculant of *R. radiobacter* SZ4S7S14

carbohydrates were used as carbon source. What concerns to a nitrogen source, yeast extract was most energetic and effective substrate for both production and biomass yield of the strain. In this substrate after 96 h of cultivation produced 3.46 g/L bioflocculant. Furthermore, D-mannose and yeast extract contributed the highest flocculating activity of bioflocculant samples—97 and 92%, respectively.

Production dynamics of the bioflocculant in the modified medium, containing D-mannose and yeast showed that highest yield was observed at 72 h of cultivation. The

combination of D-mannose and yeast extract also contributed to high biomass yield in comparing to other carbon and nitrogen sources.

Effect of cations on the bioflocculant production and flocculating activity of the purified bioflocculant

A wide variety of metal cations, such as Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Al^{3+} , Fe^{3+} and Mo^{4+} were investigated to enhance the bioflocculant production. As

Fig. 7 FT-IR of modified bioflocculant samples. The samples were obtained after cultivation of the strain in the medium consisting of: (1) glucose + malt extract; (2) sucrose + malt extract; (3) sucrose + peptone; (4) mannose + casamino acid; (5) mannose + yeast extract; (6) sucrose + yeast extract; (7) glucose + beef extract as C/N sources

Table 1 Carbon and nitrogen source impact on bioflocculant production and flocculation rate

Carbon source	Bioflocculant production, g/L	Flocculation activity, %	Nitrogen source	Bioflocculant production, g/L	Flocculation activity, %
D-Glucose	3.46	89	Yeast extract	3.46	92
D-Fructose	2.02	77	Peptone	3.41	88
Sucrose	3.51	90	Tryptone	2.87	86.5
Maltose	2.767	86	Malt extract	1.65	74
D-Mannose	5.41	97	Beef extract	2.89	87.2
			Casamino acid	2.5	80

experiments showed, Mo^{4+} , Mg^{2+} , Mn^{2+} and Fe^{2+} considerably contributed to highest bioflocculant yield in glucose/yeast extract containing medium. Of these four actions, the Mo^{4+} (tested as Na_2MoO_4 salt) effect on the bioflocculant production was higher than that of other cations (Mg^{2+} , Mn^{2+} , and Fe^{2+}) (Fig. 8a). In Mo^{4+} containing glucose/yeast extract medium, the strain produced 3.86 g/L bioflocculant or, 11.5% higher than that of without Mo^{4+} .

The role of these cations further was investigated in the flocculating activity of the purified bioflocculant. Among these cations, Ca^{2+} and Al^{3+} enhanced the flocculating activity of the bioflocculant–81 and 76.5%, respectively (Fig. 8b). As stated by Levy et al. (1992) bivalent and trivalent cations enable to decrease negative charge on both the biopolymer and the particles, whereas Li et al. (2009) and Manivasagan et al. (2015) reported that in the presence of Ca^{2+} , Al^{3+} and Fe^{3+} flocculating

activity of bioflocculants, produced by *Bacillus licheniformis* and *Streptomyces* sp. was enhanced. In our experiments, Ca^{2+} contributed to the enhanced flocculating activity of the bioflocculant, produced by *R. radiobacter* SZ4S7S14.

pH and temperature impact

Initial pH of the medium before autoclaving was 7.3. After cultivation of the strain at 30 °C and 150 rpm for 3 days (72 h), pH dropped from 7.3 to 5.1–5.2. In all media, after changing both carbon and nitrogen sources, pH varied between 7.3 and 5.1. This indicated that carbon and nitrogen sources do not considerably alter pH of the medium. But further changing pH was crucial in both biomass and bioflocculant yield. In low pH, from 2 to 3.5, the strain failed to grow and biomass yield was the lowest in comparing to other variants. The same was observed in

Fig. 8 Effect of cations on the biofloculant production (a) and flocculating activity of the purified biofloculant (b)

elevated alkalinity. In pH >8, the strain exhibited weak growth and development, therefore the biofloculant production was low.

Apart from substrates, cations, and pH, the temperature is also a crucial factor in the production of biofloculants, like other secondary metabolites (Xia et al. 2008; Li et al. 2015). Maximum activity of enzymes occurs also at an optimum temperature (Xia et al. 2008). In this study, the strain exhibited enhanced growth and the biofloculant production at 28–30 °C. As a rule for most endophytic and rhizospheric microorganisms, these temperature intervals are optimal. The strain failed to yield the cell biomass and the biofloculant synthesis in lower (>25 °C) or elevated (>35 °C) temperature intervals. It was reported that for biofloculant production by bacteria and their consortia, an optimal temperature is 28–30 °C (Xiong et al. 2010; Yang et al. 2012; Nwodo et al. 2014; Li et al. 2015).

Discussion

In recent years, interest in synthesis, production optimization, chemical characterization and practical application of biofloculants increased several times, since they are potential candidates to replace traditional flocculants, biologically safe, biocompatible and are based on cost-effective microbe biotechnologies (Gong et al. 2008; Lian et al. 2008; Yang et al. 2012). Available literature reports and discusses also a wide variety of microorganisms involved in the biosynthesis of biofloculants, screening for the active producer, the correlation between biomass, cultivation time, synthesis efficiency and impact of physico-chemical factors such as substrate (C/N sources, cations),

pH, the temperature of the overall process. This research methodologically followed the earlier reports involving a new bacterial endophyte *R. radiobacter* SZ4S7S14, isolated from medicinal plant *Ferula songorica*, and new approaches had taken to elucidate some points of biofloculant production, particularly possible modification of the biopolymer structure.

Earlier Li et al. (2009) reported the involvement of *R. radiobacter* F2 strain to produce a novel biofloculant in co-culturing with *Bacillus sphaericus* F6 strain. The optimal initial pH, culture temperature and agitation speed for a maximal yield of biofloculant by the consortia were 7–8, 30 °C and 140 rpm for 24 h. In a current report, a new strain *R. radiobacter* SZ4S7S14 researched as a potential producer of a new biofloculant.

The first and foremost requirement for each producer of a particular product is efficiency and yield of the metabolite. In this context, the strain was active than earlier reported producers of biofloculants. Xiong et al. (2010) list some bacterial strains available in the literature. Maximum biofloculant yield by *B. licheniformis* CGMCC2876 was 2.93 g/L (Xiong et al. 2010). Besides, co-cultivation of bacterial strains increased production of biofloculants. Nwodo et al. (2014) reported the enhanced synthesis of biofloculant by a consortium of *Streptomyces* and *Cellulomonas* in the modified medium, in which the biofloculant yield reached up to 4.45 g/L. In our investigation, the maximum production of biofloculant after 72 h of incubation was 5.41 g/L in the modified D-mannose/yeast extract medium. The correlation between biofloculant production, cultivation time and flocculating activity can differ among different microorganisms (Manivasagan et al. 2015). The production of biofloculant by *B. licheniformis*

(Shih et al. 2001) and *Streptomyces* sp. (Manivasagan et al. 2015) reached its maximum after 96 h, whereas strain *B. licheniformis* CGMCC2876 produced maximal amount of bioflocculant after 48 h (Xiong et al. 2010). For maximum growth and bioflocculant production, an optimal temperature range was 28–32 °C, in which produced 5.5 g/L bioflocculant. Further increasing temperature (especially $t > 35$ °C) resulted in reducing both cell biomass yield and bioflocculant production. Over 40 °C, the cell growth sharply limited and sharp drop in bioflocculant production was observed. From the obtained results, it can be concluded that for enhanced production and cell biomass yield the strain should be cultivated in D-mannose/yeast extract medium containing Mo^{4+} with pH between 6.4 and 7.3 at 28–32 °C.

In essence, bioflocculants are either microbial exopolysaccharides (Rasulov et al. 2016a) or exopolysaccharides associated with the protein (Manivasagan et al. 2015). Consecutively, bioflocculants can be composed of monomeric units such as monosaccharides and non-saccharide components (acetyl, pyruvil, succinyl groups) or inorganic substituents (e.g., phosphate, sulfate groups) (De Philippis and Vincenzini 1998). Current research showed that the bioflocculant consists of 76.2% total carbohydrates, 6.53% uronic acids and 12.5% proteins (after cultivation the strain in the ISP2 medium). Changing carbon and nitrogen sources considerably affected the total carbohydrate and protein content in the bioflocculant. The highest protein content—18.5%, was observed in the mannose/yeast extract supplemented medium, or 1.45 times higher than that of in ISP2 medium. For a maximal yield of bioflocculant by the consortium, consisting of *R. radiobacter* F2 and *B. sphaericus* F6 required glucose and urea as C/N sources (Li et al. 2009). C/N sources considerably affected the total carbohydrate and protein content of the bioflocculant, obtained by cultivating of *R. radiobacter* SZ4S7S14 in the mannose/yeast extract medium proportionally decreased, down to 72.3 and 5.6%, respectively. The similar case was observed with other carbon/nitrogen sources also. The analogical finding was reported in our earlier report (Rasulov et al. 2016b). FT-IR spectra of the bioflocculant samples of *R. radiobacter* SZ4S7S14 obtained in different carbon/nitrogen sources partially confirmed above-mentioned changes (Figs. 6, 7). The bioflocculant samples exhibited slightly varied spectra (Fig. 7). This evidence was basically observed at absorption maximums of certain functional groups, such as –OH or –NH in the carbohydrate ring, C–H asymmetrical stretching vibration typical to carbohydrates. For example, characteristic intensive peak –OH in the carbohydrate ring appeared in the region between 3412.66 and 3447.67 cm^{-1} , and it can be due to quality and quantity

change of the functional group. Moreover, it was found that changing of the bioflocculant's structure directly impacts on flocculating activity in some extent. The bioflocculant obtained after cultivation the strain in mannose and yeast extract supplemented medium, exhibited highest flocculating activity. The stretch vibrations of –OH groups of the bioflocculant (produced in mannose/yeast extract containing medium) appeared at 3447.67 cm^{-1} , whereas the lowest flocculating activity was observed in the bioflocculant with the stretch vibrations of –OH groups at 3421.10 cm^{-1} (sucrose/malt extract containing medium). It is believed that carbon/nitrogen sources primarily take part in the formation of cell structure and providing energy for the cell metabolism. But in current research, the chemical modification of final metabolite, basically total carbohydrates, and protein content was observed. In other reports, the microbial exopolysaccharides modification on a monomeric level had been discussed (Ozturk and Aslim 2010). Ozturk and Aslim (2010) reported a structural modification of exopolysaccharide of cyanobacterial strains, namely *Synechocystis* sp. under salt stress.

This finding helped to conclude that the bioflocculant of *R. radiobacter* SZ4S7S14 might be structurally modified during the metabolic activity of the strain and C/N source might alter the structure of the bioflocculant.

Author's contributions BAR participated in the design of the study, carried out the experiments and drafted the manuscript. YHL isolated the strain and did phylogenetic analysis, OAM, LL, MX, JBM participated in the evaluation of some chemical and physical properties of the bioflocculant. WJL supervised the overall study. All authors read and approved the final manuscript.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interest.

The ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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