Production and Characterization of Exopolysaccharide Produced by Alcaligenes Faecalis B14 Isolated from Indigenous Soil

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Abstract

Exo-polysaccharides (EPS) are environment friendly natural polymers secreted by microorganisms in the surrounding medium. Due to the presence of unique structural composition, EPS shows diverse applications such as in food formulations, pharmaceutical, and cement based construction industry, etc. In the present investigation, the bacteria producing higher exopolysaccharide was screened among bacteria isolated from indigenous soil samples. The characterization of bacteria was done by biochemical and 16 S rDNA analysis. The investigation was done to determine the optimal variables of nutritional and environmental conditions to get maximum EPS production from the isolated bacteria. Glucose, yeast extract and MgSO\textsubscript{4} enhanced the EPS yield at 30\textdegree C incubation temperature. Structural elucidation of the exo-polysaccharide was done using different spectroscopic techniques like \textsuperscript{1}H NMR, FTIR, and GC-MS. The monomeric composition of EPS contains glucose, rhamnose, glucuronic acid and mannose similar to welan gum. Exo-polysaccharide showed good emulsification property and viscosity that indicated its possible use as an additive in food industry.

Keywords: Exo-polysaccharide; Alcaligenes faecalis; isolation; emulsifying activity.
1. Introduction
Exopolysaccharides (EPSs) are high molecular weight, biodegradable polymers biosynthesized by a wide range of bacteria (Vijayabaskar et al., 2011). Microbial derived EPSs have advantages over traditionally used polysaccharides from plant source and seaweed-derived gums, the later are easily affected by environmental factors. EPSs show different properties like, thickening, gelling, emulsifying, etc., and in view of these properties EPS find wide range of applications in food, pharmaceutical, other industries (Nwodo et al., 2012). Although few microbial EPSs like xanthan, sphingans, cellulose etc. gain commercial importance, more interest have developed among researchers to isolate novel EPS from bacteria. Because bacteria offers wide diversity of exocellular substances with characteristic composition and properties, that provide opportunities for the development of new commercial product with improved properties. The yield of EPS varies in different strains. The production largely depends on the substrate composition and environmental conditions (Rabha et al., 2012). Thus, the optimization of fermentation process is required to achieve maximum yield of EPS. Keeping in view of the industrial importance of EPSs, present work deals with the isolation and screening of bacteria producing EPS from indigenous soil. Also study was done to determine the optimum process parameters and functional properties.

2. Materials and Methods
2.1 Isolation and identification of bacteria
Isolation of bacteria producing mucous colonies, from the sub-soil samples (collected from Barnala, Punjab, India) was performed by standard method (Benson 2001), on nutrient agar plates (at 30 °C for 24 hrs). Following the same procedure, sixteen bacteria were pure cultured and stored at -20°C as stock culture. Method of Holding & Collee (1971) was used for biochemical identification of the isolated bacteria coded as B14. Chromous Genomic DNA isolation kit (Chromous Biotech, Pvt. Ltd., India) was used for 16S rDNA sequencing of isolated bacteria. The phylogenetic tree was constructed using the neighbour-joining algorithm and similarity matrix was calculated by the method of Jukes and Cantor (Wiley et al., 1991 and Bruno et al., 2000).

2.2 Fermentation
Basal medium includes (% w/v) - glucose (2%), yeast extract (0.1%), peptone (0.3%), di-hydrogen potassium phosphate (0.2%) and magnesium sulphate (0.01%). The screening of different nutritional and physical parameters was done for EPS production. The EPS was recovered by the method of Banik & Santhiagu (2006), with slight modification. The broth was heated at 80 °C for 15 min, cooled to room temperature and pH was neutralized. The broth was centrifuged at 10,000 × g for 10 min to separate the cells. Isopropanol was added to the supernatant kept at 4 °C for 24 hrs to precipitate the EPS. The EPS was further purified by Sevag reagent (chloroform: n-butanol 4:1, v/v). The purified EPS was then lyophilized.
2.3 Analytical assay
Total carbohydrate of the EPS was assayed with phenol sulfuric acid method (Dubois, 1956). Uronic acids were assayed using carboxyl method of Dische (1962), and methyl pentoses by the method of Dische & Shettles (1948). Hydrolysis of the sample was done by the method of Kang et al. (1982) and then structure was elucidated using H’NMR spectroscopy (Bruker Avance II 400 NMR) and FT-IR spectrometer (RX-IFTIR, PerkinElmer, USA). For GC-MS analysis, the methylation of the EPS before hydrolysis was done by the methods of Hakomori (1964). An aldito acetate derivative of the methylated and hydrolyzed sample was prepared by the method of Sassaki et al. (2005). Similarly derivatization of monomers (glucose, lactose, mannose, arabinose, rhamnose, galactose, and xylose) was done. The samples were injected directly into GCMS (GC-2010, Shimadzu, Japan).

2.4 Functional properties:
The emulsification activity of EPS was estimated by the method of Cooper & Goldenberg (1987) and measurements were made after 24 h by using the following equation.

\[
\text{Emulsion index} = \frac{\text{height of the emulsion layer}}{\text{total height}} \times 100
\]

The viscosity of EPS solution (0.2 to 1.0% in deionized water; pH7; 25 °C) were measured using Brookfield viscometer, (model LVF Brookfield Engineering Laboratory, Stoughton, MA, USA).

3. Results and Discussion
3.1 Isolation and identification of bacteria
Sixteen bacteria were isolated from different soil samples and were screened for the EPS production on basal medium. A bacterium producing higher EPS among the isolated bacteria were selected for further study. The characteristics of bacteria is shown in Table 1, results were examined as per Bergey’s Manual of Determinative Bacteriology (Holding and Shewan, 1974). The 16S rDNA sequencing (Figure 1) confirmed the isolate as *Alcaligenes faecalis* B14 (accession number-KF035058).

<table>
<thead>
<tr>
<th>Test performed</th>
<th>Observation</th>
<th>Test performed</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Staining</td>
<td>Negative</td>
<td>Growth on Burk’s medium</td>
<td>Positive</td>
</tr>
<tr>
<td>Catalase test</td>
<td>Positive</td>
<td>Starch hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Positive</td>
<td>Urea hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td>Methyl Red test</td>
<td>Positive</td>
<td>Gelatin hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td>Vogues- Proskauer test</td>
<td>Negative</td>
<td>ONPG hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td>pH Observation</td>
<td></td>
<td>Temperature (°C)</td>
<td>Observation</td>
</tr>
<tr>
<td>6</td>
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<td>25</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
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</tr>
<tr>
<td>10</td>
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<td>45</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>Negative</td>
<td>55</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Figure 1: 500bp DNA ladder; 1: PCR amplified 16S rDNA of isolated bacteria (a); phylogenetic tree (b).

3.2 Optimization of physical and nutritional parameters
The effect of various physical and nutritional process parameters is shown in Figure 2. The yield of EPS increased from 0.25 mg to 0.85 mg as the pH of the medium increased from 5 to 7 and then decreased the EPS yield (Figure 2a). This may be due to changes in H\(^+\) ion concentration in the medium (Shivakumar and Vijayendra, 2005). The maximum EPS yield was observed at 30 °C and 100 rpm agitation speed (Figure 2a). Variation in agitation from 100 rpm affected the homogeneity of the culture broth which reduced mass transfer of nutrients and oxygen, thus influenced the yield of EPS (Kuntiya et al., 2010). Also with the increase of inoculum size (1-5%; v/v), the yield was increased. Above this level the production decreased because increased cell density consumes the substrate for cell growth than the production of EPS. Similar trend was reported, with gellan production from Pseudomonas elodea (Lim et al., 2003). Carbon source is required growth and EPS production by bacteria. Glucose, used as a carbon source in the medium gave highest yield of EPS followed by sucrose, maltose, and lactose. Yuksekdag & Aslim (2008) found maximum EPS yield from L. delbrueckii subsp. bulgaricus and S. thermophilus in a medium containing glucose. Among the nitrogen source, the maximum EPS yield were observed with yeast extract followed by peptone, NH\(_4\)NO\(_3\) (ammonium nitrate) and (NH\(_4\))\(_2\)SO\(_4\) (ammonium sulphate). Also with yeast extract high EPS production by Bacillus subtilis has been reported (Razack et al., 2013). Among mineral ions, magnesium sulphate (MgSO\(_4\)) gave maximum EPS yield (Figure 2b). MgSO\(_4\) has also provided maximum EPS by Xanthomonas campestris DSMZ 19000 (Enshasy et al., 2011). The ions influence the catalytic activities of enzymes involved in growth and EPS yield.
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Figure 2: Effect of physical (a) and nutritional parameters (b); on EPS production

3.3 Structural analysis of exopolysaccharide

Total carbohydrates in EPS were found to be 63%, glucuronic acid 9% and rhamnose 4.5%. Kang et al. (1982) reported 11.6-14.9% of glucuronic acid, rhamnose 33-37% in (S-130) welan gum composition. In 1D $^1$H NMR spectrum (Figure 3a), the signals at δ 4.54, 4.76, 4.82 and 5.12 were observed for anomeric protons which reveal the tetrasaccharide nature of EPS. The proton signals at δ 4.76, δ 4.54 and δ 1.29 indicates the presence of β-D-glucuronosyl, β-D-glucosyl and -CH$_3$ (rhamnose) residues. Similarly, anomeric proton signals at δ 4.55, 4.75, 5.03, 5.15, 5.28, 5.40, and 1.3 were observed for welan gum (Kumar et al., 1996). In FTIR spectrum (Figure 3b), the peak
region at ~1745 cm\(^{-1}\) is characteristic for carbonyl group indicating C=O stretching. The strong bond between 3600-3200 cm\(^{-1}\) indicates presence of O-H group. The signal at 2855 and 2923 cm\(^{-1}\) indicates the presence of C-H stretching. The absorption at 1200-900 cm\(^{-1}\) was observed and is due to stretching vibrations of C-C, C-O-C and C-O which is characteristic of carbohydrates (Singthong et al., 2005). The presence of different functional groups revealed a typical heteropolymeric nature of the sample. Similar FTIR peak range was observed for WL-26 from *Sphingomonas* sp. (Jia et al., 2012). In GC-MS chromatogram, the peaks at retention time (RT, min) 11.4, 16.4 and 17.4 were obtained and compared with (RT) obtained using monomers standards. These were identical with RT for rhamnose, mannose and glucose, thus confirmed their presence in EPS. Also similar RT at 11.5, 16.5 and 17.2 were observed for rhamnose, mannose and glucose in published literature (Li et al., 2010).

### 3.4 Functional properties

Emulsifiers are the agents that stabilize the oil and water mixture and have effect on textural properties of products when used during food processing. The emulsifying activity of 57% was observed with hydrocarbon, n-dodecane. The emulsification activity of 50%, 52.6%, 75% has been observed with hexane, toluene, and tetradecane for EPS from *Salipiger mucosus* A3 (Llamas, 2010). The commercial applicability of EPS also depends on its aqueous properties. The results (Table 2) indicated that with increase in concentration of the EPS, the viscosity was increased causing the molecule to move slowly in the solution.
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Figure 3: $^1$H NMR (a) and FTIR (b) spectrum of exopolysaccharide

Table 2: Viscosity of exopolysaccharide at different concentrations.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of exopolysaccharide (%)</th>
<th>Brookfield viscosities, (Cp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>240</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>580</td>
</tr>
<tr>
<td>3</td>
<td>0.6</td>
<td>1200</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>1620</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>1940</td>
</tr>
</tbody>
</table>

4. Conclusion
The isolate coded as B14 produced highest EPS were identified as *Alcaligenes faecalis* B14. Glucose, yeast extract and MgSO$_4$ were found as the best carbon, nitrogen and mineral salt source respectively for EPS production. The optimum physical parameters for EPS production were: temperature: 30°C; pH-7, inoculum concentration: 5% and agitation speed 100 rpm. The EPS have functional properties like good emulsification activity, viscosity. Thus, EPS have potential application to serve as an ingredient in food. The research for more detailed studies of the EPS functional properties will be carried out envisaging its use in food industry.
References


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