Physico-chemical characters of exo polysaccharide from the halotolerant Virgibacillus sp. isolated from the solar salt pan

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Abstract

Exopolysaccharide producing bacterial isolate was screened from the solar salt work and identified as Virgibacillus sp. The effect of pH, sodium chloride, temperature, carbon sources, and amino acid were screened for the growth of this organism to enhance the production of polysaccharide. The optimum sodium chloride requirement for growth of the isolated was found to be 9\% NaCl. The growth was optimum at pH 7.0 and 40 °C. Among the carbon source tested, lactose, galactose and fructose significantly enhanced the growth of organism. The amino acids such as, arginine, proline and tyrosine positively influenced the growth of Virgibacillus sp. The purified extracellular polysaccharide was soluble in distilled water. The emulsification index of extra polysaccharide was 77.27\% which contain 11.26\% ash. The viscosity of this sample was found to be 2.91 × 10\textsuperscript{-3}. The physio – chemical properties of extrapoly saccharide produced by this organism has potential application.

1. Introduction

Exopolysaccharides (EPSs) are extracellular polymers which are produced by the microorganisms such as bacteria, fungi and blue-green algae (Amjres et al., 2014). EPSs are mainly composed of a mixture of organic substances, such as carbohydrates (Cescutti et al., 1999; Sutherland and Kennedy, 1996), protein, and deoxyribonucleic acids (Tsuneda et al., 2001; Zhang et al., 1999). EPSs possess hyperglycaemic, anti-oxidation, hypolipidemic, anti-tumor, anti-radiation and many biological activities, which are hotspots of active research on active principles of drugs and health food (Chen et al., 2004; Li et al., 2006; Tseng et al., 2008). The EPS of microorganisms possess a wide variety of physical, structural, rheological and other properties, which makes it renewable sources of various biotechnological applications (Kavita et al., 2011). In recent years, attempt has been made to explore valuable EPS due to its various biotechnological applications. The wide structural, physical and rheological diversity and other unique properties of EPS produced by bio film-forming bacteria make it industrially and biotechnologically important. EPS is widely used in food-industry, which act as stabilising, viscosifying and emulsifying agent (Mishra and Jha, 2013).

Microorganisms are best suited for EPS production than algae or plants, exhibiting better growth rates and being more amenable to optimize, manipulation of the process conditions for enhancing growth and EPS production (Moreno et al., 1998). Moreover, the high cost of the nitrogen, carbon sources such as sucrose, glucose and fructose have direct impact on EPS production costs, which directly limits the market potential of EPS (Kumar et al., 2007; Sutherland, 2001). The information about the concentrations of EPS is
scarce (Liu et al., 2001). Quantification of EPS is mainly dependent upon the extraction procedures (Wingender et al., 1999), physical extraction procedures include heating, centrifugation, and ultrasonication, whereas chemical extractions such as the use of ethylenediamine tetraacetic acid (EDTA), alkaline and resin. However, there is no standard extraction procedure established so far making it too difficult to compare the yield with other organisms. EPS was produced and characterized from bacteria such as Vibrio harveyi, V. alginolyticus (Bramhachari and Dubey, 2006; Bramhachari et al., 2007), Lactobacillus plantarum YW11 (Wang et al., 2015), Lactobacillus suebicus (Ibarburu et al., 2015) and the flocculation efficiency of EPS was reported from Virgibacillus sp. (Cosa et al., 2011).

In recent years novel EPS was isolated from saline habitats, because most of the reported EPS so far was non-halophilic in nature. Because of the hypersaline environment, they may feasibly harbour unusual microorganisms of biotechnological interest. EPS was characterized from the organisms such as Halomonas eurihalina (Quesada et al., 1990), H. ventosae (Martínez-Cánovas et al., 2004) and Alteromonas suebica (Martínez-Checka et al., 2005) and these EPS showed potential interest as jellifying, emulsifying, viscosifying and metal-binding properties (Mata et al., 2006; Mata et al., 2008). EPS yield and the composition of monosaccharides greatly depend on the candidate species, their process conditions and media compositions (Salazar et al., 2009). In this study, a novel microbial biopolymer is described. Along with the optimization of fermentation process for the growth of organism, the chemical composition of the EPS has been studied.

2. Objective of Research

Exopolysaccharides from Lactobacillus sp. are widely used in the food industry as stabilizing, viscosifying, emulsifying and emulsifying agents, due to their characteristic physical and rheological properties. However, the studies on EPS from Virgibacillus sp. are comparatively less. Therefore, the present study was aimed to produce EPS in optimized condition and study its biochemical characters.

3. Methodology

Isolation and characterization of EPS producing Virgibacillus sp. from solar salt work.

The soil sample was collected from solar salt pan, Puthalam, Kanyakumari District, Tamilnadu. The samples were serially diluted and plated on Zobell marine agar plates. The potent EPS producing isolate was retained by observing for good mucoid colony morphology (Fusconi and Godinho, 2002). The selected EPS producing bacterial isolate was identified based on their morphological and biochemical characteristic features (Garrity et al., 2001).

Production of exopolysaccharide in submerged fermentation

The identified Virgibacillus sp. was subjected for the production of EPS in submerged fermentation. This organism was inoculated in the medium containing Zobell marine broth and incubated for 4 days at 37 °C. The culture was centrifuged at 12,000 rpm for 10 min at 4 °C. The EPS was precipitated from the cell free extract by adding double volume of methanol. Further, the precipitate was collected by centrifugation at 25,000 rpm for 20 min. The final pellet obtained was dried at 60 °C and weighed.

Optimization of the growth of Virgibacillus sp.

To study the effect of pH on the growth of organism, the medium pH was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 by the addition of 1.0 N HCl/NaOH. To study the effect of temperature on its growth, the culture was incubated at various temperatures ranging from 30 – 60 °C. To study the effect of NaCl on the growth of organism, the organism was inoculated and incubated into the medium containing 7 - 12% sodium chloride. To the culture medium, 1% carbon sources (fructose, galactose, lactose, maltose, and sucrose) were added to elucidate the effect of carbon sources. To study the effect of amino acid on the growth of organism, the amino acids such as arginine, cysteine, histidine, proline, tryptophan, and tyrosin were supplemented. The EPS was precipitated as described previously and the final pellet obtained was dried.

Purification of EPS

The four days culture broth was precipitated from the supernatant by the addition of double volumes of methanol. This procedure was repeated four times and dried at 60 °C. The EPS sample was further analysed by High-performance liquid chromatography (HPLC) at 400 nm for 10 minutes. Characterization of EPS

Emulsification test

Emulsification assays were carried out according to the method of Cooper and Goldenberg (1987). EPS was dissolved in 5.0 ml double distilled water and mixed with 5.0 ml of hydrophobic substrate (Kerosene oil) in the series of test tubes, which were vortexed to homogeneity and incubated for 24 h at 4 °C. Emulsifying activity was expressed as the percentage of the total height occupied by the emulsion after 25 h.
Graphical abstract

Virgibacillus sp.

Exopolysaccharide Production

Optimization of EPS production

Characterization of exopolysaccharide

Figure 1:

Table 1:

<table>
<thead>
<tr>
<th>pH</th>
<th>Incubation period (day)</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>0.2 0.3 0.39 0.39 0.4 0.37</td>
</tr>
<tr>
<td>6</td>
<td>0.3 0.58 0.55 0.54 0.53 0.44</td>
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<tr>
<td>7</td>
<td>0.65 0.67 0.58 0.57 0.59 0.6</td>
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<tr>
<td>8</td>
<td>0.38 0.67 0.69 0.6 0.54 0.49</td>
</tr>
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<td>9</td>
<td>0.18 0.29 0.76 0.43 0.42 0.4</td>
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Table 2:

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<th>Temperature (°C)</th>
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<tr>
<td>30</td>
<td>0.18 0.56 0.5 0.49 0.33 0.32</td>
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<tr>
<td>35</td>
<td>0.2 0.22 0.32 0.35 0.33 0.3</td>
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<tr>
<td>40</td>
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<td>45</td>
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<tr>
<td>50</td>
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<td>60</td>
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**Table 3:**

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<th>Sodium chloride (%)</th>
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<td>7%</td>
<td>0.49</td>
</tr>
<tr>
<td>8%</td>
<td>0.23</td>
</tr>
<tr>
<td>9%</td>
<td>0.22</td>
</tr>
<tr>
<td>10%</td>
<td>0.06</td>
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<tr>
<td>11%</td>
<td>0.11</td>
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<tr>
<td>12%</td>
<td>0.11</td>
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**Table 4:**

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<th>Carbon sources (1%)</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Fructose</td>
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<tr>
<td>Galactose</td>
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<tr>
<td>Lactose</td>
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<td>Maltose</td>
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<td>Sucrose</td>
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**Table 5:**

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<th>Aminoacid</th>
<th>Incubation period (day)</th>
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<tr>
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<td>Arginine</td>
<td>0.33</td>
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<tr>
<td>Cysteine</td>
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<tr>
<td>Histidine</td>
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<td>Proline</td>
<td>0.17</td>
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<tr>
<td>Tryphtophan</td>
<td>0.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.52</td>
</tr>
</tbody>
</table>

**Viscosity test**

For the analysis of viscosity of sample, EPS was dissolved in double distilled water and analyzed at 25 °C using a Viscosity meter (Model – D21). Determinations were made through a shear – stress range between 0.15 and 1.5 pa (Mata et al., 2006).

**Solubility test**

Solubility test was performed using water, chloroform, acetone, ethanol, methanol, benzene, and ethyl acetate. 2.0 ml of these solvent was taken into a sterile test tube, to this 0.1 gm EPS was added individually. The contents were mixed thoroughly and allowed to stand for few minutes. Later, the tubes were observed for the solubility of EPS.

**Bio film assay**

Bio film assay was carried out by crystal-violet-adhesion assays as described by O'Toole and Kolter (1998). For this assay, overnight cultures were inoculated at a ratio of 1:100 into fresh medium and were then grown for 40 h in a 96-well polystyrene microtitre plate. Cell growth was determined by analyzing its absorbance at 600 nm. Cell growth was determined by analyzing its absorbance at 600 nm. Bio film was measured by discarding the medium, rinsing the wells with water and staining and bound cells with crystal violet. The dye was dissolved in ethanol and absorbance was determined at 540 nm using a microtitre plate reader.

**Estimation of ash content**

Total ash content in EPS was estimated by gravimetric method. About 2 gm of EPS was placed in a tarred crucible, weighed accurately and slowly carbonized using a muffle furnace at 575 °C until the sample turned into white ash to constant weight. The ash content was weighed and the percentage of the total ash was calculated. The weight of the residue, which represented the ash content, was recorded and the results are given as percentage of the dry weight of polysaccharides.

**4. Results and Discussion**

The bacterial strain was isolated from the solar salt pan by Zobell marine agar plates. The isolated organism was found to be Gram’s negative, rod – shaped. The optimum sodium chloride requirement for the growth of isolate was found to be 9% NaCl (w/v). It could grow moderately even in the absence of sodium chloride, indicating its halotolerant nature. Colonies are regular to irregular, translucent and flat, and milky white in colour. The pH range for the growth is 6–8, with an optimum at 7.0. Microbial growth occurs under aerobic conditions. H₂S production-negative, Nitrate reduction-positive, gelatine and casein are hydrolysed, Tween 20 and Tween 80 are hydrolysed, Voges–Proskauer reaction is negative. Indole is not produced by this bacterial isolate. The strain was identified as *Virgibacillus* sp. and showed mucoid colonies. As per earlier report the
exopolysaccharide producing colonies show mucoid appearance.

Many polysaccharide producing bacteria were isolated from various sources and EPS production was optimized and characterized. EPS production by the microorganism has been generally considered as a mechanism of self-protection by the bacterial or fungal isolates during unfavourable conditions such as high acidity, phage attack and dehydration (Ruas-Madiedo et al., 2002). The production of EPS can be affected by process conditions, such as composition of culture medium, pH, aeration efficiency and temperature. In the present study, the halotolerant Virgibacillus sp. was isolated from solar salt pan for the production of EPS. Various physical and nutrient factors were optimized for the growth of Virgibacillus sp. Considering the medium components on EPS production, the bacterial growth was optimized for the production of EPS. To improve the growth of Virgibacillus sp., it was inoculated at various pH ranges and growth was found to be maximum at pH 7.0 (Table 1). The acidic stress inhibited bacterial growth and stimulate the production of EPS. With the aim of detecting the effect of temperature on the growth of organism, this organism was inoculated and incubated at various temperatures and the optimum temperature for the bacterial growth was recorded at 40 °C (Table 2). The medium temperature is considered the most important factor which significantly influenced on the growth and EPS production of S. thermophilus (Kumar et al., 2007). In the present study, the bacterial growth was found to be high at 9% NaCl (Table 3).

In microbial fermentation, the carbon source functions as a source of both constituent cellular material and energy (Kumari et al., 2008). It was previously reported that carbon source, nitrogen source, and ions are significant factors on EPS production (Cerning, 1990; Vaningelgem et al., 2004). Results revealed that the supplementation of carbon sources in the growth medium increased the growth of Virgibacillus sp. Among the carbon source tested, lactose significantly enhanced the growth of organism (Table 4). The quality and quantity of EPS production is significantly influenced by medium composition and fermentation conditions (Sutherland, 1998). Hence, the optimization of growth condition is significantly important. In Halomonas sp. CRSS, acetate was found to be the most significant carbon source for the production of fructoglucon polysaccharide (Poli et al., 2004), and glucose and the mixture of peptone and yeast extract was found to be optimum for the production of a very high molecular EPS. In Lactobacillus casei CG11, glucose was found to be the suitable source for the production of EPS (Cerning et al., 1994). In Sphingomonas pancinobilis, supplementation of soybean enhanced higher productivity of EPS (West and Strohfus, 1998).

Supplementation of amino acid is one of the important factors for the production of EPS. In the present study, the amino acids such as arginine, cysteine, histidine, proline, tryptophan and tyrosine were supplemented along with the growth medium. Among these sources, tyrosine significantly influenced the growth of Virgibacillus sp. (Table 5). In general the amino acid supplements generate metabolic energy and involve in decarboxylation of the substrates. Recently, D-amino acids have been identified as the significant factor influencing EPS production (Zhang et al., 2014).

The extracted EPS was further fractionated with ice cold methanol and the purity of EPS was evaluated with High Performance Liquid Chromatography (HPLC). One major peak was obtained had showed its purity (Figure 1). The purified EPS was used for further characterization studies. The solubility of EPS in various solvents depends on the distribution of hydrophobic and hydrophilic residues in the structure of EPS. If the EPS contains more hydrophilic residues, then it is easily soluble in the polar solvents like water. Results revealed that the EPS of Virgibacillus sp. contains more hydrophilic residues in its structure. The dried EPS was not soluble in methanol, benzene, ethyl acetate, chloroform, acetone and ethanol. The isolated EPS was soluble only in water. This result was in consistent with the earlier report by Borgio et al. (2009) stated that the soluble nature of EPS in water and insoluble nature in organic solvents, which showed the general properties of EPS. The EPS solution in water was very clear, homogeneous liquid with light yellow. In the present study, the emulsifying index of the EPS was found to be 77.27%. The emulsifying index of EPS in the present study was significantly higher than other reports. Huang et al. (2012) observed the emulsifying index of R. miluonense CC-B-L1, B. seminalis CC-IDD2w and E. adhaerens CC-GSB4 and the emulsifying index was 66, 64 and 60%, respectively. In the present study, 11.26% ash content was detected from Virgibacillus sp. However, this ash content was less than that of Codium tomentosum. The ash content observed in this study was higher than the polymer isolated from T. fuciformis (Gao et al., 1996b).

**Conclusion**

Bacterial extracellular polymers are abundant and ubiquitous in extreme environment where they serve essential functions that enhance the survival of microbes. In halotolerant environment, the EPS
may provide protection against high salinity, pH and also at high temperature. The isolated *Virgibacillus* sp. growth was optimized for the production of EPS. The physio-chemical characters of EPS were examined and the properties were found to be novel. By examining its physio, chemical-properties, it is possible to exploit this organism for the commercial production of EPS.

**Research Highlights**

Extracellular polymeric substances (EPS) was isolated and characterized from *Virgibacillus* sp.

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We received no funding for this work.

**Author’s Contribution and Competing Interests**

All authors contribute equally. The authors declare that there is no conflict of interests regarding the publication of this paper.

**References**


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