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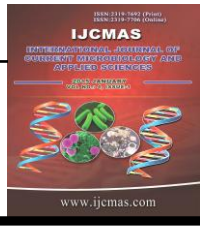

Original Research Article

Production of exopolysaccharide by an osmotolerant, thermostable and metal resistant *Bacillus subtilis*

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A B S T R A C T

Conventionally polysaccharide was obtained from plants and seaweeds. Wide repertoires of novel polysaccharides are also obtain from microorganisms. Increasing attention is being paid to these molecules because of their bioactive role and their extensive range of potential applications in pharmaceutical and agricultural industries. In present study exopolysaccharide producing *Bacillus subtilis* strain was newly isolated from sugar industry waste. The strain shows mucoid growth on MGYP agar plate. Mucoidness indicates the presence of polysaccharide which was confirmed by the analytical methods. The identification of isolate was performed by using its morphological, cultural, biochemical characteristics. It was further confirmed by the 16s rRNA gene sequencing. The isolate was optimized at three different pH values (4, 7, and 9), maximum growth was observed at pH 7. It shows ability to grow at wide range of temperature *i.e.* from 25^{o}C to 55^{o}C. It also shows ability to grow at high concentrations of sugar *i. e.* up to 25%. The isolate was found to be resistant to several antibiotics. In addition to antibiotic resistance the isolate also showed multiple resistance to metals like Pb, Ni, Co and Zn. Organism produces EPS in two distinct forms *i.e.* ropy EPS and loose slime that is excreted into the surrounding and capsular EPS that remained adhered to cell surface creating discrete covering. The production of EPS was performed by the batch culture method; which was recovered by the ethanol precipitation. The solubility of EPS in different polar and non-polar solvents was studied. The carbohydrate and protein content of EPS was determined. It was found that 495 µg/ml and 380 µg/ml respectively.

Keywords

Exopolysaccharide, *B. subtilis*, Osmotolerant, Thermostable, Metal resistance

Introduction

Microbial exopolysaccharides (EPS) are the polymers that consists principally carbohydrates and are excreted by some bacteria and fungi outside of their cell wall. EPS is occur in two forms depending on their location, i. e. capsular polysaccharide (capsule) where the polymer is closely associated with the cell surface and as the slime polysaccharides that are loosely associated with the cell surface. For the cells, EPS plays important role in protection against the desiccation, toxic compounds,

bacteriophages, osmotic stress and permit the adhesion to the solid surfaces and biofilm formation. Microbial polysaccharides with important mechanical properties have significant impact in commercial applications. In the continuing search for novel, natural water soluble polysaccharides, particular attention has been directed in a recent year to the production of extra cellular polysaccharides of microorganisms. The first microbial polysaccharide to be commercialized was dextran; it was used as a blood plasma extender and in other applications. Increasing attention is being paid to these molecules because of their bioactive role and their extensive range of potential applications in pharmaceutical as anti-angiogenic (Matou *et al.*, 2005) or antiviral agents (Arena *et al.*, 2009) and in agriculture and various other industrial areas (Sutherland, 2003). The microorganisms can produce large amount of polysaccharides in the presence of surplus carbon sources. Some of these polysaccharides (*e.g*. Glycogen) serve as storage compound. The polysaccharides excreted outside by the microbes, referred to as exopolysaccharides, are of commercial important. The exopolysaccharide may be found in association with the cells or may remain in the medium (Sutherland, 2003).The microbial polysaccharides may be neutral or acidic in nature. Acidic polysaccharides possessing ionized groups such as carboxyl, which can function as polyelectrolyte, are commercially more important. Exopolysaccharides are high molecular weight polymer with charged functions groups and possesses both adsorptive and adhesive properties due to the presence of charged moieties; exopolysaccharides ideally serve as a natural ligand source, providing binding sites for the charged particles or molecules including metals (Suresh & Mody, 2009).

Materials and Methods

Isolation of EPS producing organism

The samples of sugar industry waste from Shirpur (Maharashtra) having high sugar content was screened for the isolation of EPS producing microorganisms. These samples were inoculated in MGYP broth (For 100ml, sucrose-20g, peptone-0.5g, yeast extract-0.3g, malt extract-0.3g). The broth was incubated for 24 h at 37^{o}C.On next day; the organisms from broth were streaked on MGYP agar plates. Few colonies show mucoid growth, which were isolated and further streaked on MGYP agar plates.

Identification of isolate

The isolate was identified on the basis of its morphological characteristics by Gram staining and capsule staining. Cultural characteristics like size, shape, colour, margin, elevation, consistency, opacity were studied. Biochemical characteristics like Catalase and amylase enzyme production tests were performed for the identification of the isolate. It is further confirmed by 16s rRNA gene sequencing.

pH, temperature and sugar concentration optimization

Microorganisms has the ability to grow at their optimum values of pH, they shows maximum growth at that pH value. Optimization of pH for the growth of isolate was performed at three different pH values i.e. at 3, 7 and 9. Whereas the optimization of temperature for the growth of isolate was performed at wide range of temperature i.e. 4^{o}C to 55^{o}C. Optimization of sugar concentration at 5%, 10%, 15%, 20%, and 25%was also performed for the growth of isolate.

Antibiotic sensitivity test

The isolate was studied for the sensitivity to different antibiotics like carbenicillin, ampicillin, chloramphenicol, cotrimazine, streptomycin etc. This test was performed by using disc diffusion method and octadisc method.

Heavy metal resistance

Generally, heavy metals affect the growth of microorganisms i.e. they inhibit the growth of microbes by using oligodynamic power. Isolate was grown in presence of various concentrations of heavy metals like Pb, Ni, Co, Cu, Ni, and Zn, in order to check its sensitivity to them.

Production of EPS

For the production of exopolysaccharide (EPS), inoculum was developed by inoculating the isolated organism into 100 ml of MGYP broth. After the inoculum preparation the 8% inoculum was inoculated into 250 ml of MGYP broth for the production of EPS by batch culture method. The cultural conditions were maintained for 24 h. EPS was recovered by using ethanol precipitation. The fermented broth was centrifuged at 8000 rpm for 20 min. The supernatant was collected and cell pellets were discarded. Then the chilled ethanol was added in 2:1 (ethanol: supernatant) proportion. By using glass rod the solution was stirred and stored at room temperature for overnight. On next day, the EPS layer was observed at bottom. The upper layer in flask was discarded and EPS (crude) was collected and allowed to dry it.

Solubility of EPS in different solvents:

The solubility of EPS in different solvents is depends on the distribution of hydrophilic and hydrophobic residues in the structure of polysaccharide. If the amounts of hydrophilic residues are more in the structure of EPS then it is easily soluble in a polar solvent like water. The excess amount of hydrophobic residues in the structure of EPS allows it to soluble in non-polar solvents like acetone, chloroform and benzene.

Small quantities of dried EPS pellets were taken in different eppendroffs, then2 ml of solvents such as water, acetone, chloroform, ethanol, methanol, benzene, formic acid, ammonia, carbon tetrachloride, hexane, tetra hydrofuranwas added into respective eppendroffs.Mixed thoroughly using a vortex mixture and observed for pellet formation.

Determination of carbohydrate and protein content

Determination of carbohydrate content of EPS was done by using phenol sulphuric acid method. In this method sugars undergo dehydration in the presence of sulphuric acid to furfural or hydroxymethyl furfural that condenses with phenol to form a yellowish-orange compound with absorption maxima at 490 nm.

Determination of protein content of EPS was done by using Folin-Lowery method. In this method protein reacts with FolinCiocaltaeu reagent to give a coloured complex. The colour so formed due to the reaction of alkaline copper with the protein as in the biuret test and reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of colour depends on the amount of these aromatic amino acids present in the protein.

Result and Discussion

Isolation EPS producing organism

For the isolation of EPS producing microorganisms MGYP medium is used. Initially the samplewas inoculated in the MGYP broth for the enrichment of the microorganisms. The broth becomes viscous after 24 h of incubation period, which indicates the presence of exopolysaccharide. The organisms from broth were taken on MGYP agar plates, and finally the organisms showing mucoid growth on MGYP agar plates (Fig.-1) were selected as exopolysaccharide producers.

Identification of isolate

The identification of isolate was done by using morphological, cultural, and biochemical studies (Table-1). The potent isolate was straight rod, Gram positive and endospore forming organism. It was aerobic, motile, amylase and catalase positive. On the basis of the results of morphological, physiological and biochemical tests the strain was belongs to *Bacillus* sp. After performing 16s rRNA gene sequencing studies, it was confirmed that, the isolated strain was identified as *Bacillus subtilis.*

pH, temperature and sugar concentration optimization

The isolate showed maximum growth at pH 7 (Table-2). It also showed ability to grow at wide range of temperature i.e. upto 50^{o}C, which indicates its thermostable nature, but it showed maximum growth at 37^{o}C (Table-3). The isolate was examined for the growth at various sugar concentrations. The organism showed ability to grow at wide range of sugar concentration i.e. upto 25%, which proves its Osmotolerant nature (Table-4).

Antibiotic sensitivity test

Antibiotic sensitivity test was performed by disc diffusion method. Isolate showed resistance to carbenicillin, ampicillin, chloramphenicol and cotrimazine.

Heavy metal resistance

Isolated organism showed multiple resistances to heavy metals like Pb, Ni, Co and Zn.

Production of EPS

Production of EPS was performed by the batch culture method. After a complete incubation period of 24 h, viscous growth was observed in the broth. Recovery of the EPS was carried out by ethanol precipitation. After treating the broth with chlilled ethanol the precipitate formed, which is recovered by centrifugation. The pellet collected, dried and by grinding, the fine powder was prepared.

Solubility of EPS in different solvents

EPS is soluble only in the water and insoluble in all other non polar solvents (Table-5). This idicates the abundent distribution of hydrophilic residues in the structure of polysaccharide. It could be further confirmed by the detail structural analysis.

Determination of carbohydrate and protein content

The concentration of protein in the exopolysaccharide was determined by Folin Lawry method and it was found to be 380µg/ml. Whereas, The concentration of carbohydrate in the exopolysaccharide was determined by phenol sulphuric acid method and it was found to be 495µg/ml.

Table.1 Morphological, cultural, and biochemical characteristics of an isolate

Characteristic	Result
Size	2mm
Shape	Rod
Margin	Entire
Elevation	Flat
Opacity	Opaque
Consistency	Smooth
Gram character	Gram positive
Spore staining	Spore former
Catalase enzyme	Positive
Amylase enzyme	Positive

Table.2 Optimization of pH for the growth of an isolate

pH	Growth
4	-
7	++
11	+

Table.3 Optimization of temperature for the growth of an isolate

Medium	Temperature	Growth
Liquid	4˚C	–
Liquid	37˚C	+++
Liquid	45˚C	++
Liquid	50˚C	+
Liquid	55˚C	–
Solid	4˚C	–
Solid	37˚C	+++
Solid	45˚C	++
Solid	50˚C	+
Solid	55˚C	–

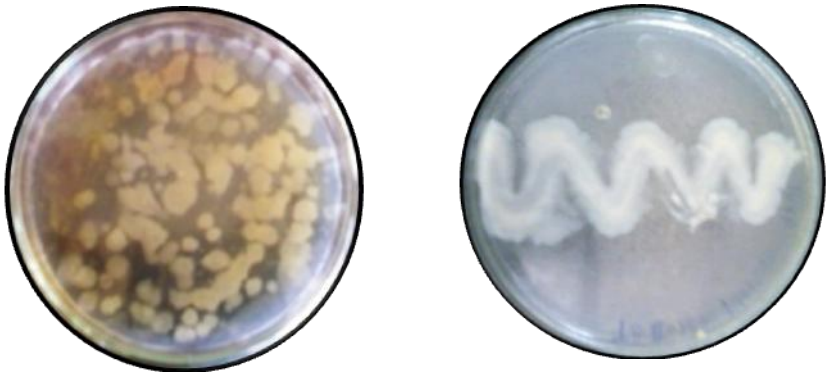
Table.4 Optimization of sugar concentration for the growth of an isolate

Sugar concentration	Growth
5%	+
10%	+
15%	+
20%	++
25%	+

Table.5 Solubility of EPS in different solvents

Solvent	Solubility
Water	Soluble
Chloroform	Insoluble
Hexane	Insoluble
Benzene	Insoluble
Ammonia	Insoluble
Formic acid	Insoluble
Methanol	Insoluble
Tetrahydrofuran	Insoluble
Ethanol	Insoluble
Carbon tetra chloride	Insoluble

Fig.1 Isolated mucoid colonies of *B. subtilis* on MGYP agar plates



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