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Enhanced production and partial characterization of an extracellular polysaccharide from newly isolated *Azotobacter* sp. SSB81

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ABSTRACT

A strain was selected by its highest extracellular polysaccharide (EPS) production ability compare to other isolates from the same rhizospheric soil. The selected strain was identified by 16S rDNA sequencing and designated as SSB81. Phylogenetic analysis of the gene sequence showed its close relatedness with *Azotobacter vinelandii* and *Azotobacter salinestris*. Maximum EPS (2.52 g l^{-1}) was recovered when the basal medium was supplemented with glucose (2.0%), riboflavin (1 mg l^{-1}) and casamino acid (0.2%). The EPS showed a stable viscosity level at acidic pH (3.0–6.5) and the pyrolysis temperature was found to be at 116.73 °C with an enthalpy (ΔH) of 1330.72 Jg^{-1}. MALDI TOF mass spectrometric result suggests that polymer contained Hex_5Pent_3 as oligomeric building subunit. SEM studies revealed that the polymer had a porous structure with small pore size distribution indicating the compactness of the polymer. This novel EPS may find possible application as a polymer for environmental bioremediation and biotechnological processes.

1. Introduction

Azotobacters are non symbiotic diazotrophic bacteria generally found in neutral or slightly alkaline soils, including aerially transported dust and mostly abundant in plant rhizosphere and phyllosphere region. Members of the genus *Azotobacter*, are free living, gram negative, aerobic and heterotrophic in nature (Garg et al., 2001). *Azotobacter* sp. is well known to produce large quantities of extracellular polysaccharides (EPS), commonly apparent as large mucoid colonies during isolation from soil habitat (Vargas-García et al., 2002). EPS provide protection to cells against desiccation and predation by protozoans or phage attack (Looijesteijn et al., 2001). This polysaccharide also protects the penetration of toxic metal ions inside the cell (Aleem et al., 2003) and nitrogenase against high oxygen concentration environment (Sabra et al., 2000; Soto-Urzúa and Baca, 2001). Moreover, microbial exopolysaccharides have found multifarious applications in food, pharmaceutical, other industries (Kumar et al., 2007; Freitas et al., 2009) and also participates in interaction between plants and bacteria (Leigh and Coplin, 1992; Mandal et al., 2007). Furthermore, microbial EPS is an important source for detoxification of metals and wastewaters (Ozdemir et al., 2003; Aguilera et al., 2008).

Exopolysaccharide production by *Azotobacter* has been traditionally investigated among different species and strains. The compositions of EPS have been found to be different within the diversity of the strains (Sutherland, 1994). The EPS of *Azotobacters* have excellent viscosifying properties and physiological activity different from natural gums and synthetic polymers. They are highly susceptible to biodegradation in nature and less harmful than synthetic polymer. The EPS production of the present isolate is better than other isolates and also stable at acidic pH (3.0–6.5), indicating its potential industrial importance. The aim of the present work was to standardize the physico-chemical conditions for higher yield of the EPS and analysis of its physiological properties.

2. Methods

2.1. Isolation and identification

Bacteria were isolated from rhizospheric soil of well populated forest following the method of Brown et al. (1962). Screening and selection of the strain have been made on the basis of their EPS production ability in nitrogen-free glucose Burk's medium (glucose, 20 g; K_2HPO_4, 0.2 g; K_2SO_4, 0.1 g; $MgSO_4$, 0.2 g; NaCl, 0.2 g; $CaCO_3$, 5 g; Na-molybdate, 0.01 g; water, 1000 ml) (Vela and Rosenthal, 1972). The selected strain was identified by 16S rDNA sequencing and designated as SSB81. For 16S rDNA sequencing, total genomic DNA was extracted from the isolate following the method Sambrook et al. (1989). Amplification of 16S rDNA gene by PCR using universal primer 27F [5′-AGAGTTTGATCCTGGCTCAG-3′] and 1492R [5′-GGTTACCTTGTTACGACTT-3′]. Using these

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primers (20 pmol each) and 200 ng genomic DNA of the isolate in a 50 μl reaction buffer containing 2 mM dNTP, 1.5 mM $MgCl_2$ and 5 units Taq polymerase (Bioline, USA) and PCR was performed in a thermocycler (ABI, USA). The PCR conditions were an initial denaturation for 1.5 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 7 min. PCR products were analyzed by 1.0% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV-transilluminator. The PCR amplified DNA was eluted from gel and purified by QIA quick gel extraction kit (QIAGEN). Sequencing was done using Bigdye terminator kit (ABI) and same primers (used for PCR) in an automated DNA sequencer (ABI model 3100, Hitachi). The sequence was then compared with those available in the GenBank databases using BLAST (Pearson and Lipman, 1988) and phylogenetic analysis was done using *MEGA* version 3.1 program by NJ (Neighborhood Joining)/MP (Maximum Parsimony) method (Kumar et al., 2004).

2.2. Culture conditions

EPS production and the growth of the organism were tested in 100 ml nephelometric flasks containing 25 ml of N_2 free basal glucose medium with a shaking of 180 rpm. Temperature, pH and time have been optimized for better yield of EPS. Different carbon sources were added individually to the basal medium except control to determine the effect of carbon dose on EPS production by the isolate. Individual effect of different nitrogen sources (ammonium sulphate, sodium nitrate, potassium nitrate, ammonium chloride, L-tryptophan, glutamic acid, casamino acid and L-asparagine) and vitamin sources (Ca-pantothenate, riboflavin, cyanocobalamine, L-biotin and pyridoxal phosphate) with effective carbon source on EPS production was checked. All the supplements were added to the medium after filter sterilization in aseptic condition. Growth of the organism was determined in terms of optical density by spectrophotometer at 620 nm.

2.3. Estimation of extracellular polysaccharide

Cultures were centrifuged (10,000g for 10 min) and ammonium acetate (1 M) was added to the supernatant. EPS was precipitated in the supernatant by addition of double volume of isopropanol. The precipitate was then dissolved in deionized water. Yields of EPS were estimated by the phenol sulphuric acid assay (Dubois et al., 1956) against freshly prepared nitrogen-free basal glucose medium as control. Glucose in the culture medium was estimated calorimetrically following the method of Bernfeld (1955).

2.4. Viscosity measurement

For determination of viscosity, a 2.0% solution of EPS was prepared in deionized water. Measurements were taken in a viscometer (ViscoStar R, Spain) with 30 rpm at 25 °C. pH of the EPS solution was adjusted using 1 N HCl and 1 M NaOH solution.

2.5. Differential scanning calorimetry (DSC)

The pyrolysis pattern of the EPS was investigated using a differential scanning calorimeter (Pyris Diamond DSC, Perkin Elmer, USA). The EPS (5 mg) was enclosed in an aluminium vessel and its energy level was scanned from 40 to 350 °C with a temperature increase of 10 °C min^{-1}.

2.6. MALDI-TOF mass spectrometry

The EPS was partially degraded with 100 mM trifluroacetic acid and the acid-resistant material was removed by diluting the digest with 80% aqueous ethanol. The ethanol-soluble oligosaccharide was concentrated under N_2. One milligram of oligosaccharide was dissolved in 1 ml deionized water. Prior to MALDI analysis, sample was mixed with equal volume of 2,5-dihydroxybenzoic acid (10 mg/ml) as matrix. MALDI analysis was performed on an Applied Biosystem Voyager-DE PRO MALDI ToF mass spectrometer with a nitrogen laser (337 nm) operated in an accelerating voltage (20 kV). Each spectrum was collected in the positive ion reflector mode as average of 100 laser shots. The data was externally calibrated using angiotensin and ACTH (Applied Biosystem, USA). Reproducibility of each spectrum was checked 20 times from duplicate prepared sample.

2.7. Scanning electron microscopy (SEM)

Cultures were centrifuged (10,000g for 5 min) and ammonium acetate (1 M) was added to the supernatant. EPS was precipitated in the supernatant by addition of double volume of isopropanol. The precipitate was then washed three times with aqueous 1% osmium tetroxide for 2 h at 4 °C. The samples were then dehydrated by sequential transfer to increasing concentrations of acetone up to anhydrous acetone, critical-point dried from CO_2, and sputter coated with gold. The morphology of the EPS have been observed under a scanning electron microscope (VEGA/TESCAN), with an accelerated voltage of 10–20 kV.

3. Results and discussion

3.1. Identification and phylogenetic analysis

Using conserve primers of 16S rDNA, a 1.5 kb DNA was amplified by PCR from the genomic DNA of isolated bacteria and 468 nucleotides from its 5′ end were sequenced. Phylogenetic analysis of this obtained sequence (accession no. EU652411) showed close relationship of SSB81 with *Azotobacter vinelandii* and *Azotobacter salinestris* (Fig. 1).

3.2. Optimization of extracellular polysaccharide production

The strain (SSB81) reached its stationary phase of growth after 36 h in N_2 free basal glucose medium. Production of EPS by the strain was started at the beginning of its growth and reached maximum at 30 h of its exponential phase, when the utilization of glucose was maximum (Fig. 2). Interestingly, the EPS production was suddenly dropped after 30 h, it seems to be the utilization of EPS as carbon source for their growth. The strain showed a maximum level of EPS production at pH 8.0 followed by pH 7.0. Earlier Dhanasekar et al. (2003) reported that *Azotobacter* likes to grow at alkaline pH 7.5–8.0. In nitrogen-free basal medium, $CaCO_3$ was used as a buffer to provide an alkaline reaction for the growth of *Azotobacter*. The optimum temperature for growth and EPS production has been found to be at 30 ± 2 °C. *Azotobacter* utilized glucose and cultivated in a batch reactor, was reported to have optimum temperature at 30 °C (Dhanasekar et al., 2003). This is general trends for EPS production by *Azotobacter* sp., though a temperature of 34 °C was optimum for some strains have also been reported (Chen et al., 1985).

Among the carbon sources glucose was found best for EPS production (Fig. 3). EPS production was also studied at various concentration of glucose and it is found that maximum production occurred at 2% (w/v) glucose that remains unchanged up to 4%, this is due to the structural simplicity of glucose and utilized easily than other tested carbon sources. However, in case of sucrose and lactose, growth and EPS production was also stimulated but higher concentration of those carbon sources were inhibitory for