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Bacillus circulans exopolysaccharide: Production, characterization and bioactivities

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ABSTRACT

A bacterium with the ability to produce appreciable amount of exopolysaccharide was isolated from slimy layer of coconut. 16S rDNA analysis identified the organism as *Bacillus circulans*. EPS production was observed at all stages of culture growth and reached maximum of 0.065 mg/ml by 96 h, which on further incubation started to decrease. Response Surface Methodology using Box Behnken design has shown the influence of sucrose which was found to be directly proportional to exopolysaccharide production with production reaching 1.09 mg/ml. HPLC analysis identified the presence of glucose, mannose, fructose and verbascose and NMR analysis confirmed the presence of glucose, mannose and galactose. Even though the extracted *B. circulans* EPS did not show appreciable anti-bacterial or anti-fungal activity, it exhibited appreciable antioxidant, anti-inflammatory and anti-tumor activity.

1. Introduction

Exopolysaccharides (EPS) are chemical compounds which are synthesized by various microbes by fermenting different carbon sources and are secreted outside the cell wall [1–3]. A large number of gram negative and gram positive bacteria, yeasts and many fungi produce biopolymers using glucose, sucrose, molasses, hydrolyzed starch, methanol and different hydrocarbons [4].

Exopolysaccharides are essential to the biological success of most bacteria living within the biofilm in the varied natural environments. Exopolymers have an important role in bacterial adhesion and are associated with irreversible adhesion. Moreover, they can coat surfaces enhancing or avoiding bacterial colonization [5]. The EPS play an important role in concentrating nutrients from flowing water making it accessible for the microbes. Further, the matrix protects the bacteria from toxic chemicals and predators [6]. The polymers are predominantly composed of carbohydrates, but many contain various other components such as proteins, DNA, and glycolipids [7]. Exopolysaccharides occur widely and especially among prokaryotic species of both free-living (saprophytes) and those pathogenic to humans, animals and plants (parasites) [8].

The material properties of exopolysaccharides have revolutionized the industrial and medical sectors due to their functional applications and prospects [9]. Phenomenal demand for natural polymers for various industrial applications has led to a vibrant interest in exopolysaccharide production by microorganisms. Bacterial exopolysaccharides encompass a broad range of complex chemical structures and consequently exhibit different properties [10].

The biotechnological use of EPS includes Environmental, Clinical, Nutritional and Cosmetic application [11,12]. EPS are also used as bio flocculants, bio absorbents, encapsulating materials and heavy metal removing agents [13,14]. The exo polysaccharides have proven results on cholesterol lowering function, anti-tumor, immune modulating and probiotic effects [15]. The possible application of these polysaccharides is in human health has been listed by Iyer et al. [16].

All the reported or successful polysaccharides with anti-diabetic or anti-cancer property are used in form of syrups or food additives. They cannot be supplied in the form of drugs because they have pronounced activity only at higher concentrations. Further, the exact concentration at which the compound is active is not clear. The best example is PSP and PSK of *Coriolus versicolor* which is taken in form of syrups [17].

The main objective of the work is to isolate a bacterial strain capable of producing appreciable amount of EPS, optimize the medium for maximum production of EPS, characterize the EPS using various instrumental analyses and estimate its biological activity which will qualify the EPS as a potential compound that can be used in medical formulations.

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2. Materials and methods

2.1. Microorganisms

Spoiled vegetables, slimy layer of coconut, fermented flour, sediments from different oil cans, cloth amended with oil, ready mix pastes, sewage isolates, scrapings or swabs from catamaran were screened for the presence of exopolysaccharide former. Initially the samples were plated on nutrient agar medium and highly mucoid and non haemolytic colonies were selected for EPS production and extraction, eliminating the well-known EPS former *Lactobacilli* genus. A loop full of selected mucoid colonies were inoculated in nutrient broth with 1% of glucose and incubated overnight. The culture broths were screened for its grams reaction, motility and capsule formation. Simultaneously the isolates were subjected to biochemical characterization that helps in the identification of the genus.

2.2. Culture conditions

Bacillus circulans for EPS production were grown in a basal salt solution (BSS) having the following composition (g/100 ml): sucrose 1.0, yeast extract 0.5, sodium di hydrogen phosphate 0.3 g and casamino acid 1 ml/100 ml. pH of the medium was adjusted to 7.5 with 1 N NaOH. The medium was sterilized by autoclaving for 20 min at 121 °C and was inoculated with 2% (v/v) of 18 h old culture grown in the same medium at room temperature on a rotary shaker at 150 rpm.

2.3. EPS extraction/quantification

Cells from the culture broth were removed by centrifugation at 10,000 × *g* for 10 min. The clear supernatant was pressure filtered through nitrocellulose membrane with a pore size of 0.45 μm. 3 volumes of ice cold ethanol was added to the clear supernatant and incubated at 4° C overnight. A fine network in the upper layer of solvent that appeared was collected by centrifugation at 15,000 × *g* for 15 min. The collected pellets were purified by dialysis using a pore size of 14,000 KDa MW membranes against distilled water. Extracted, purified EPS were lyophilized and stored for future use.

The EPS was estimated by the phenol-sulphuric acid method of Dubois et al. [18]. A sample of 1 ml of the diluted solution was taken and 1 ml of conc. H_2SO_4 phenol mixture was added and placed in an ice bath for 5–15 min and the absorbance of the samples at 494 nm was determined using spectrophotometer.

2.4. Optimization of medium—Response Surface Methodology using Box Behnken design

Based on prior experiments sucrose, yeast extract, calcium chloride and casamino acid were found to be major variables in polymer production when temperature and pH were kept constant. The concentration of each component required to increase the yield of EPS production by *B. circulans* was optimized by statistical experimental design using Design expert version 7.0, Stat-Ease, Minneapolis. This method involves number of empirical techniques to evaluate the correlation of experimental factors and predict the critical concentration of dependent and independent variables.

A Box Behnken design was applied to obtain the experimental data that fits in full Quadratic polynomial model representing the response surface over a relatively broad range of parameters. The range and the levels of experimental variables investigated are presented in Table 1.

The quadratic equation

$$Y = \gamma_0 + \gamma_1 A + \gamma_2 B + \gamma_3 C + \gamma_4 A^2 + \gamma_5 B^2 + \gamma_6 C^2 + \gamma_7 AB + \gamma_8 BC + \gamma_9 AC \quad (1)$$

where Y is the measured response, A, B and C are the coded independent input variables, γ_0 is the intercept term, γ_1, γ_2, and γ_3 are the coefficients showing the linear effects, γ_4, γ_5 and γ_6, are the quadratic coefficients showing the squared effects and γ_7, γ_8 and γ_9 are the cross product coefficients showing the interaction effects.

2.5. Characterization of EPS

2.5.1. Fourier Transform Infra-red (FTIR) analysis

The major functional groups of the EPS were identified using FTIR spectrophotometer. 0.5 mg of dried sample was ground with 150 mg of KBr crystals and was made into a pellet using a hydraulic press. The pellets were subjected to FTIR analysis using PerkinElmer IR spectroscope.

2.5.2. Nuclear Magnetic Resonance (NMR) analysis

The NMR spectra were obtained on a Bruker AMX-500 instrument (500.13 MHz for ^{1}H NMR and 12.5 MHz for C ^{13}) at 70 °C. Chemical shifts were reported in PPM relative to sodium-d 4-trimethylsilyl propionate for ^{1}H and $CPCl_3$ for ^{13}C NMR spectra.

2.5.3. HPLC analysis

Free sugars were determined by a High Performance Liquid Chromatography (HPLC). A stainless steel column of 0.05 m long and 4.6 mm in internal diameter followed along with a stainless steel column 0.15 m long and 4.6 mm in internal diameter, both packed with amino propyl silyl silica gel for chromatography [3 μm] maintained at 38 °C was used. Mobile phase at a flow rate of 1.0 ml/min was prepared by dissolving 0.253 g of sodium di hydrogen phosphate in 220 ml of water and 780 ml of acetonitrile R. Refracto meter maintained at a constant temperature is used as detector (Agilent Bio HPLC Column Selection Guide). Identification of sugars was done by comparing the relative retention times of sample peaks with that of standards.

1.00 mg of the test Sample was mixed with 2 ml of water and 2.5 ml of acetonitrile with gentle heating. 0.5 ml of water was added to the sample to form the test solution. Reference solution was prepared with glucose, verbascose, mannose and galactose in 20 ml of water and 25 ml of acetonitrile with gentle heating and 5 ml of water.

2.5.4. GC–MS analysis

Twenty milligrams of the EPS were hydrolyzed with 2 ml of 2 M Tri Fluoroacetic Acid (TFA) at 100 °C for 2 h. After hydrolysis, methanol was added into the dry sample and evaporated. The hydrolysate was then subjected to GC–MS analysis (GCMS-QP2010 Ultra, Shimadzu) for the determination of the monosaccharide composition [19].

2.6. Biological activity

2.6.1. Anti-oxidant activity (DPPH method)

The DPPH free radical-scavenging activity of the exopolysaccharide fraction was determined according to the method described by Gyamfi et al. [20]. Briefly, 0.1 mM of ethanolic DPPH radical solution was prepared. The initial absorbance of the DPPH radical in ethanol was measured at 517 nm which remained the same throughout the assay. An aliquot (1.0 ml) of sample (with appropriate dilution) was added to 3.0 ml of ethanolic DPPH radical solution. Decrease in colour was measured at 517 nm after incubation in dark for