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Medium optimization for the production of exopolysaccharide by *Bacillus subtilis* using synthetic sources and agro wastes

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Abstract: Exopolysaccharides (EPSs) play an extensive role as biopolymers in the environment by replacing synthetic polymers as they are degradable, nontoxic, and produced by microorganisms. An attempt was made to optimize a medium, by the one-factor-at-a-time method, for an enhanced production of EPS from a soil isolate, *Bacillus subtilis*. The study was carried out by experimenting on various nutrients at different concentrations. EPS was precipitated using ethanol, the total carbohydrate content was determined by phenol sulfuric acid method, and functional groups were detected by Fourier transform infrared (FTIR) spectrophotometry. The finalized medium contained sucrose (20 g/L), yeast extract (5 g/L), NaCl (7 g/L), $CaCl_2$ (0.5 g/L), L-asparagine (0.05 g/L), and ascorbic acid (0.05 g/L). The carbon source was replaced with certain agro substrates, cane molasses, and rice bran. Cane molasses at a concentration of 2% gave the highest yield of 4.86 g EPS/L as compared to a medium with sucrose (2.98 g EPS/L). The effect of UV radiations on growth and synthesis was negative, decreasing the growth rate and quantity of EPS produced. Different solvents were checked for their efficiency on precipitating EPS; those other than ethanol, diethyl ether, and methanol were not able to sediment the polymer. FTIR analysis of the extracted product revealed that the polymer was made up of units of sucrose. Thus, the present study showed that the agro wastes could be an alternative for synthetic substrates, providing a way for an economical production of EPS.

Key words: *Bacillus subtilis*, exopolysaccharide, cane molasses, rice bran, FTIR spectrophotometry

1. Introduction

Microbes release polysaccharides extracellularly as exopolysaccharides (EPSs) into the environment in the form of capsules or slime. Naturally occurring polysaccharides possess a unique combination of functional properties and environmentally friendly features. They are renewable in nature, nontoxic, and biodegradable (1). Microbial polysaccharides are water soluble polymers and may be ionic or nonionic. Microbial EPSs, containing 90% or more polysaccharides (2), could be categorized into 2 broad classes: homopolysaccharides, which are compounds of single units of monosaccharide, and heteropolysaccharides, which are composed of 2 or more units of monosaccharide.

EPSs are highly important to any bacterium as a defense mechanism; to prevent desiccation (3); for adhesions by forming biofilms (4,5); and in industries as gelling agents, biosurfactants, emulsifiers, viscosifiers (6–8), biosorbents (9,10), and biologically active antimicrobials, anticancer agents, and antioxidants (11–14).

EPS is often produced at a lower temperature than is required for optimum growth (15). It also requires higher carbon content in the medium and decreased nitrogen quantity (16). Factors that could influence the production of EPS are the composition of the medium, especially carbon and nitrogen sources, and parameters like pH, temperature, and incubation time. The commercial value of EPS would be determined by the ease of production, the quality produced, the composition of the polysaccharide, and the mode of harvest. A huge variety of biopolymers such as polysaccharide, polyesters, and polyamides are naturally produced by microbes. They range from a viscous solution to plastic, and their physical properties are dependent on the composition and molecular weight of the polymer. The genetic manipulation of the microorganism opens up an enormous potential for the biotechnological application with tailored properties suitable for tissue engineering and drug delivery.

Bacillus subtilis has been selected for study due to its structural and genetic complexity. Wild strains of *B. subtilis* are capable of forming architecturally complex communities of cells known as biofilms (17,18).This organism is highly utilized for biofilm and endospore formation studies. *B. subtilis* has the capacity to transform from the motile state to the nonmotile state. In nonagitated liquid broth, the highly motile cells, swimming singly,

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accumulate as bundled chains forming pellicles. The EPS operon is believed to be responsible for the biosynthesis of an EPS that binds the chains of cells together in bundles forming biofilms. A 15-gene operon designated as yveK-T yvfA-F, later renamed epsA-O and under the control of both Spo0A and σ^H, identified as transcriptional factors, was predicted to encode products likely to be involved in EPS synthesis and export (17).

Recent investigations were carried out to produce EPSs for biotechnological applications at a lower cost. For cost-effective production, agro industrial wastes are used as substrates (19). Molasses is the final effluent obtained in the production of sugar by repeated crystallization (20). Sugarcane molasses could be a better source of carbon due to its higher content of total sugars at 48.3%.

The present study is meant to develop a medium and optimize the components for production of EPSs from *B. subtilis*. The work also focuses on the comparative study of the production of EPS using synthetic nutrients and agro wastes (rice bran and cane molasses) as carbon substrates.

2. Materials and methods

2.1. Culture condition

The organism for study, *Bacillus subtilis*, was isolated from a soil sample from the university campus by undergoing routine microbiological techniques, serial dilution, and biochemical characterization (21). Morphological identification was carried out by Gram staining, negative staining, and endospore staining. Biochemical characterization of the culture was done using a series of tests such as indole production, methyl red, Voges–Proskauer, citrate utilization, carbohydrate fermentation, casein hydrolysis, starch hydrolysis, nitrate reductase test, lipolytic activity, H_2S production, catalase, and oxidase tests (www.microbelibrary.org). Growth at various salt concentrations and different temperatures were also studied. The purified culture was stored on nutrient agar slants at 4 °C as stock for further study.

2.2. Experimental setup

2.2.1. Effect of carbon, nitrogen, NaCl, minerals, vitamins, and amino acids on EPS production of *B. subtilis*

A 24-h culture was used throughout the study. The nutrient broth was inoculated with 2% inoculum and incubated at 3 °C for 48 h.

To study the effect of carbon sources, various sugars like glucose, fructose, lactose, mannose, xylose, and sucrose were added at varying concentrations of 1%, 2%, 5%, 7%, and 10% to each flask with 100 mL of nutrient broth and inoculated with 2% inoculum. The effect of nitrogen sources was tested by adding different nitrogen sources like peptone, yeast extract, NH_4Cl, $NaNO_3$, and beef extract at concentrations ranging from 0.1% to 1.1% with an interval of 0.2%, replacing standard nitrogen sources of nutrient broth composition. The effect of salt concentrations on EPS production was checked by adding NaCl at varying concentrations from 0.1% to 1.1% at an interval of 0.2% in the nutrient broth. To observe the effect of mineral sources, salts like $CaCl_2$, $FeCl_3$, $MgSO_4$, KH_2PO_4, $CoCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, and $NaMoO_4$ were added at the concentrations of 0.01%, 0.03%, 0.05%, 0.07%, 0.09%, 0.1%, 0.5%, and 1.0% to the nutrient broth. The effect of vitamins (ascorbic acid, vitamin B1, and biotin) and amino acids (L-asparagine, L-glutamine, L-glycine, and L-cysteine) were used at concentrations of $(2.5–10 \times 10^{-3})$% at an interval of 2.5%.

2.2.2. Effect of raw substrates on EPS production of *B. subtilis*

Agricultural wastes like cane molasses and rice bran were used for the study.

Cane molasses was obtained from a sugar factory, which was pretreated for the work. Molasses was diluted with distilled water containing 2% sodium dihydrogen phosphate in the ratio of 1:1 and autoclaved (22). The solution was then left overnight for settling and the clarified molasses was used in concentrations 1%, 2%, 5%, 7%, and 10% in the broth as the substitute for sucrose.

Rice bran obtained from a local rice mill was pretreated by heating at 100 °C for 20 min in a hot air oven (23), stored in a moisture-free environment, and used at varying concentrations of 1%, 2%, 5%, 7%, and 10% as a replacement for synthetic sucrose.

2.2.3. Effect of UV radiation on EPS yield

The culture suspensions were kept under UV light at a distance of 54 cm. Each flask was exposed to UV at different time intervals ranging from 10 to 70 min with intervals of 10 (24). The culture suspensions were inoculated on agar and incubated at 37 °C for 24 h, which were later checked for the productivity of EPS.

2.3. Isolation and extraction of EPS

The culture was centrifuged at 11,000 rpm for 10 min. The supernatant collected was mixed with an equal volume of ice-cold ethanol and incubated at 4 °C for 24 h. The refrigerated solution was then centrifuged at 2500 rpm for 20 min. The obtained pellet was resuspended in distilled water, along with an equal volume of ice-cold ethanol. The solution was then centrifuged again at 2500 rpm for 20 min. The final pellet obtained was dried at 60 °C and weighed (25).

2.4. Use of different solvents for extraction

The effect of different solvents in extracting EPS from the supernatant was tested. To each 10 mL of the supernatant, an equal volume of various solvents (methanol, ethanol, butanol, isoamyl alcohol, pentanol, acetone, chloroform, diethyl ether, formaldehyde, xylene, and benzene) was

added. The tubes were incubated for 24 h at 4 °C (26). The precipitation of EPS was observed.

2.5. Determination of total carbohydrate content

Total carbohydrate content was determined by the method of Dubois et al. (27). To the dried pellet, 1 mL of 5% phenol and 5 mL of 96% concentrated sulfuric acid was added and the mixture was kept in a boiling water bath for 20 min. The optical density of the sample was read spectrophotometrically at 490 nm and total carbohydrate content was calculated, using sucrose as standard.

2.6. Fourier transform infrared spectrophotometry

A quantity of 50 mg of an EPS sample, obtained from medium with cane molasses, was added to 150 mg of potassium bromide (KBr) pellets and ground well. The powered composition was pressed into a disk using a hydraulic press (28). The disk was then used for analysis with a Fourier transform infrared (FTIR) spectrophotometer (Bruker Optics GmbH, Germany) with the wavelengths ranging from 400 to 4000 cm^{-1}.

3. Results and discussion

In the present study, EPS was extracted and isolated from soil bacterium *Bacillus subtilis*, which was confirmed by microbiological techniques and biochemical characterization. Table 1 represents the biochemical characterization of the microorganism, which produced highly mucoid colonies denoting the generation of extracellular polysaccharides.

Effects of various synthetic nutrient sources were determined in this study. At a concentration of 2% sugars, EPS was at its maximum. Among the synthetic carbon sources tested for the highest EPS generation, 2% sucrose yielded 2.66 g EPS/L (Figure 1). Maltose and fructose gave the minimum yields at 1.42 and 0.96, respectively, indicating their negligible effect on fermentation. Glucose and lactose were also able to yield a near maximum of approximately 1.8 g/L at the same concentration. Himanshu et al. reported that ratios of carbon and nitrogen sources play the most important role in cellular growth and exobiopolymer production (29). Sucrose at a concentration of 100 g/L was also found to be the best source for EPS production from *B. licheniformis* 221a, at 13.57 g EPS/L of medium (30). Sucrose, a disaccharide, upon hydrolysis produces glucose and fructose. Higher yield is obtained, since sucrose apparently acts as a precursor of EPS synthesis. As the concentration of the sugars increased above 2%, the cell growth and the yield were found to decline. This is mostly due to the elevation of osmotic pressure in the cellular system, thereby causing plasmolysis, leading to cell death (31). Various studies have been carried out to learn the effects of different carbon substrates on EPS production. A concentration of 2% maltose in the production medium was able to produce 3.5 g EPS/L from *Cordyceps jiangxiensis* (32). A maximum of 44.49 mg/L of EPS was produced from *Lactobacillus fermentum* when the medium was supplemented with 2% glucose and 0.5% whey protein concentrate (33). Sugars like fructose, lactose, glucose, and sucrose were used for EPS production in *Streptococcus thermophilus* ST1 from skim milk, yielding 64.52 mg/L, 66.39 mg/L, 69.35 mg/L, and 73.28 mg/L, respectively (34).

Various nitrogen sources were observed for their effects on EPS yield from the isolate. Organic nitrogen sources were inferred to yield a higher amount of EPS than inorganic nitrogen substrates. Yeast extract was found to produce the maximum yield of 1.38 g/L at a concentration of 0.5%. On using inorganic nitrogen sources, NH_4Cl and $NaNO_3$, the growth and yield were generally retarded when compared to use of organic nitrogen substrates (Figure 2). It was suggested that certain essential amino acids cannot be synthesized from inorganic nitrogen components (35), because of which bacterial cells might neither fully grow

Table 1. Phenotypical characterization of the soil isolate *B. subtilis*.

Characteristics	Inference
Gram staining	+
Motility	+
Capsular staining	+
Endospore staining	+
Indole production	–
Methyl red test	+
Voges–Proskauer test	+
Citrate utilization test	+
H_2S production	–
Catalase test	+
Oxidase test	–
Gelatin liquefaction	+
Starch hydrolysis	+
Casein hydrolysis	+
Nitrate reduction test	+
Lipolytic activity	+
Growth in NaCl	
• 2%	–
• 5%	+
• 10%	–
Carbohydrate fermentation test	
• Glucose	+++
• Fructose	+++
• Lactose	++
• Xylose	+++
• Mannose	+++
• Mannitol	+++
• Sucrose	+++

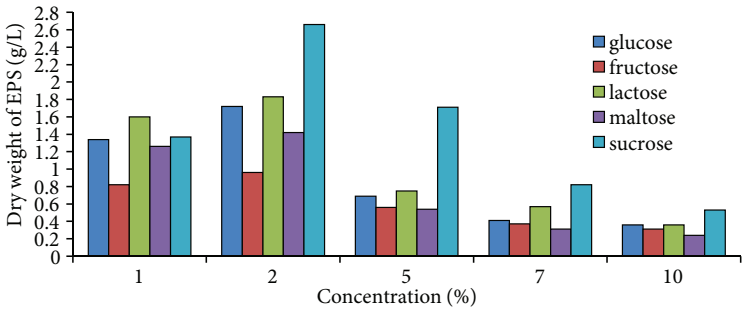

Figure 1. Effect of various carbon substrates at different concentrations.

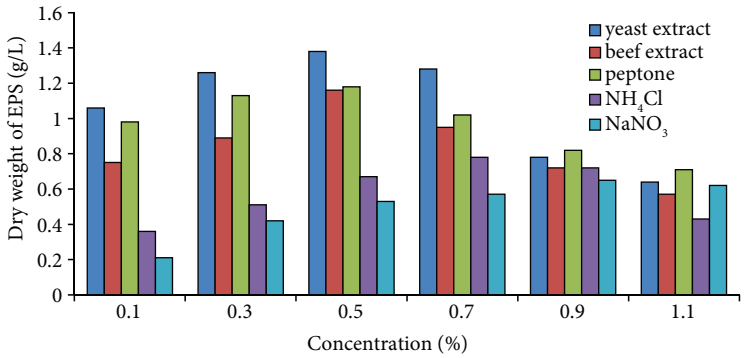

Figure 2. Effect of nitrogen sources on EPS production.

nor undergo metabolism, and hence the deterioration of EPS yield. It was also reported that the primary role of heterotrophic bacteria is classically considered to be decomposition and mineralization of dissolved particulate organic nitrogen (36). This might be an obvious cause of higher production of EPS by *B. subtilis*. As the concentration of the nitrogen sources was increased, the growth rate was found to ascend, but the mitigation of EPS production was observed. Reports suggest that nitrogen limitation and higher amounts of carbon in the medium could yield a maximum amount of EPS (37). A study showed that EPS production from *Rhizobium meliloti* was higher when the nitrogen source was in minimal quantity (38). Similarly, pullulan was generated by *Aureobasidium pullulans* when it was grown in a medium with lesser amounts of nitrogen source (39).

Salinity was an essential culture parameter for the production of higher amounts of EPS. In the present work, the highest amount of biopolymer was obtained with NaCl at a concentration of 0.7% as 1.31 g/L. With higher or lower values than the optimal concentration of 0.7% NaCl, decrease in extracellular metabolite was observed (Figure 3). Like that observed with the sugars, the changes in salt concentrations caused instability of osmotic pressure that led to detrimental effects on bacterial cells (30).

The effects of different mineral salts were studied at different concentrations and revealed that $CaCl_2$, at the concentration of 0.05%, gave the maximum yield of 1432 mg/L. At very low concentrations (0.01%, 0.03%), it did not show much effect on EPS production. It was reported that certain minerals (Ca^{2+}, Co^{2+}, Fe^{2+}, K^{+}, and Mn^{2+}) were favorable to the mycelial growth and EPS production of *P.*

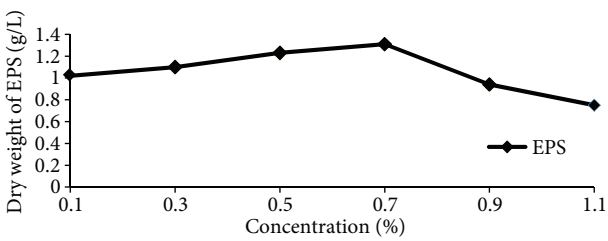

Figure 3. Effect of NaCl on the yield of EPS.

sinclairii, and as the concentration was increased, EPS was found to be increasing (40). In contrast to this, the present study showed that as the concentration was increased, growth and EPS yield were decreased. At the maximum concentration used, no growth was obtained, especially with Fe^{3+}, Cu^{2+}, and Mg^{2+} (Figure 4).

In studying the effects of amino acids and vitamins, L-asparagine showed the maximum effect on yield of EPS, followed by L-glutamine and L-glycine (Figure 5). Vitamins also showed a moderate effect on the production of EPS. The present work showed that ascorbic acid yielded the maximum EPS of 19 mg/L. Vitamins and amino acids are required for growth and extracellular product formation, and might have a significant role in the cellular metabolism, especially as precursors for EPS biosynthesis (41). A report showed that the EPS production and growth of *B. subtilis* strain 51 have been influenced markedly by methionine, leucine, isoleucine, cystine, glycine, tryptophan, and alanine (42). In contrast to this, L-cysteine showed no effect on the soil isolate of *B. subtilis* in the present work.

This study was also aimed at using different raw agricultural wastes as a carbon source, as it is the most required nutrient for EPS production. Clarified cane molasses and finely powdered rice bran were used for the work. Different concentrations of cane molasses and rice bran in the medium showed that 2% cane molasses and 5% rice bran produced the highest yields at 4.86 and 2.14 g/L, respectively (Figure 6). When compared to the yield of EPS using raw substrates with synthetic sucrose, molasses was able to yield EPS at its maximum at a lower concentration. The difference between total carbohydrate content of clarified and unclarified molasses was meager and these values were found to be 72 and 74 g/L, respectively, thus negating the issue of negative effect of pretreatment of the substrate, revealing that sucrose in clarified molasses aided in yielding higher EPS.

Molasses is effective on growth medium as it possesses high vitamin and mineral contents and also has a significant growth stimulatory effect (11). Due to its many advantages like high sucrose and other nutrient contents, low cost, ready availability, and ease of storage, molasses has been used as a substrate for fermentation production of commercial polysaccharides like curdlan, xanthan, dextran, scleroglucan, and gellan (43). Previous experiments reported on the use of molasses for EPS production. *B. cereus* B-11 was able to produce biopolymers in a medium containing molasses waste-water, replacing glucose and yielding 500 mg/L (44). A fungus, *Mucor rouxii*, produced 87% EPS in medium with 3% beet molasses (45). *A. pullulans* produced 16.9% pullulan in molasses medium with initial sugar concentration of 50

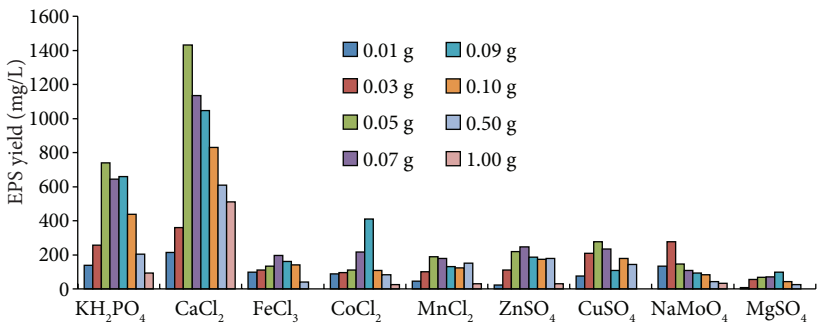

Figure 4. Effect of minerals on the production of EPS.

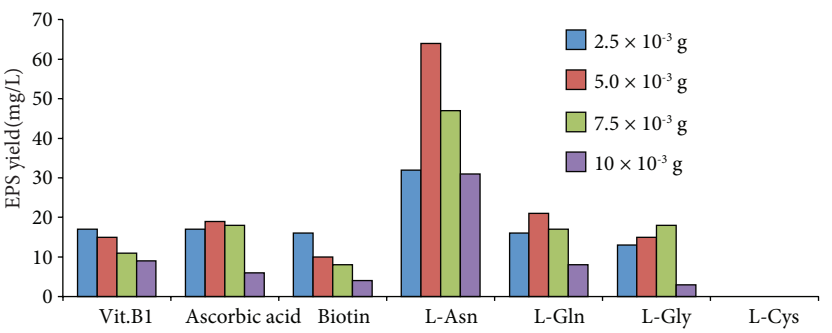

Figure 5. Effect of vitamins and amino acids on EPS production. L-Asn = L-asparagine, L-Gln = L-glutamine, L-Gly = L-glycine, L-Cys = L-cysteine.

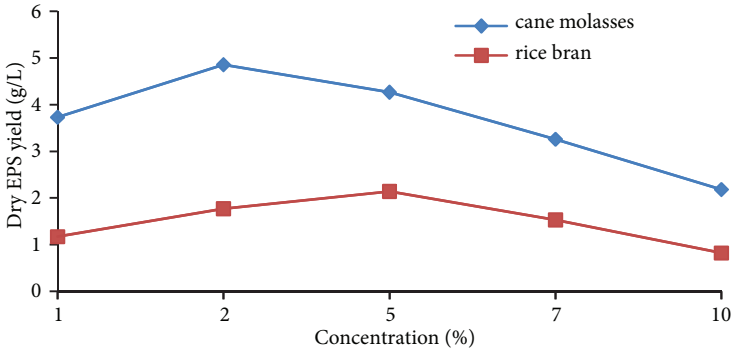

Figure 6. Graph representing EPS yield (g/L) using cane molasses and rice bran.

g/L at pH 7.5 (46). *Azotobacter* was able to produce 7.5 mg EPS/mL of medium with 2% beet molasses (47).

The experiments with rice bran as carbon substrate were carried out similarly to the experiments with cane molasses, which resulted in a yield of EPS of 2.14 g/L, which was maximum at a concentration of 5% rice bran (Table 2). Rice bran was found to be inefficient since the quantity of EPS was less even at higher concentrations when compared to the production using molasses (Figure 6). This clearly indicated that the growth rate and EPS synthesis was high due to the presence of a higher content of sucrose in cane molasses than in rice bran, at 590 mg/118 g of crude rice bran (www.nutritiondata.self.com). Rice bran, because of its high cellulose content, cellulose being a polysaccharide, might have been difficult for microbes to utilize as a nutrient. Similarly, a study showed that a lower yield was obtained when rice bran was used as the carbon substrate, at 2.4 g EPS/kg, to produce EPS from *A. pullulans* when compared to the yields of EPS using cassava starch and wheat bran (48). *Sinorhizobium meliloti* produced 11.8 g/L of medium with 20% rice bran hydrolysate at 96 h of fermentation (49).

UV radiations have an adverse effect on EPS production. Upon exposure to UV rays at regular time intervals of 10 min to 70 min, deterioration of EPS was observed gradually and cells exposed for 70 min produced a meager quantity of EPS (Figure 7). It was also found that the size of the cells was reduced upon exposure for a prolonged period (i.e. 30–70 min) when compared with unexposed cells. An EPS operon is believed to be responsible for the biosynthesis of the EPS that binds chains of cells together (50). *B. subtilis* is reported to be highly accessible to manipulation by techniques of classical and molecular genetics (50).The present study indicated that UV radiations might have had an effect on genetic and internal biochemical alterations, thereby drastically changing the metabolism.

Various solvents were tested for their efficiency in extracting the biopolymer from the culture. In this study, the standard solvent was ethanol and it was compared with other solvents. Ethanol precipitated EPS but required 24 h for the process and overnight incubation, whereas methanol sedimented EPS instantaneously upon addition to the culture supernatant. Diethyl ether and acetone also effectively precipitated, but only after 24 h. Other solvents (butanol, propanol, isoamyl alcohol, toluene, xylene, benzene, and chloroform) showed no effect on precipitation. A white interface was formed at the juncture of solvent and supernatant, which apparently consisted of lipids and proteins. This study thus revealed that diethyl ether and acetone can also be used for the isolation of exopolymers from bacteria.

Analysis of the composition of the EPS isolated in the present study by FTIR spectroscopy revealed that the polymer is composed of units of sucrose, and it was compared with reference sucrose. The absorption peak at 3494.91 indicated the presence of OH groups. The ester

Table 2. Comparative study of the yield of EPS using nutrient broth, medium with sucrose as the synthetic carbon source, medium with rice bran, and medium with sugarcane molasses as the agro-carbon substrates.

Variables	Nutrient broth	Optimized medium with sucrose (2%)	Optimized medium with rice bran (5%)	Optimized medium with sugarcane molasses (2%)
Dry cell mass (g/L)	1.59	2.31	2.87	3.54
Dry EPS (g/L)	1.44	2.98	2.14	4.86
Total carbohydrate content (%)	53	76	63	89

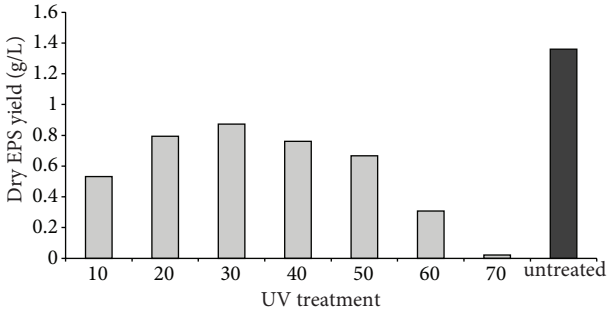

Figure 7. Comparative graph illustrating the yield of EPS (g/L) by *Bacillus* cells exposed to UV at various time intervals (purple) along with unexposed cells (green).

group was assigned to the peak at 1665.00. The vibrational stretch of the C-O-C group was found at the absorption peak of 1058.48.

Various reports have shown that several kinds of EPS were produced by *Bacillus* spp. *Bacillus polymyxa* produced EPS in the presence of sucrose (61 g/g of sugar) in 31 h of cultivation (38). An acidic type of polysaccharide was produced by a variety of *Bacillus* strains. A polysaccharide containing glucose, galactose, fucose, and glucuronic acid is produced by *B. subtilis* (51). *Bacillus* strain CMG1447 also produced a high molecular weight acidic heteropolysaccharide containing glucuronic acid, galactose, mannose, and rhamnose (51).

In the present study, an attempt was made to produce EPS from soil isolate *B. subtilis*. The data showed that cane molasses could be a better alternative for exopolymer production, replacing the synthetic carbon substrate, sucrose. EPS yield was at its maximum, 4.86 g/L, when cane molasses concentration was 2% at 48 h of fermentation. The medium thus formulated could be cost-effective as an agro waste was being used as the major nutrient source. Further studies could be carried out in elucidating the structure of the biopolymer and involving EPS in various biological activities like immunomodulatory effects, anticancer activities, and antimicrobial effects.

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