

Exopolysaccharide from *Bacillus mojavensis* DAS10-1; Production and Characterization

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Among group of bacteria screened for exopolymer production, *Bacillus* species DAS10-1 producing exopolysaccharide was detected. The bacterial strain was isolated from indoor air of Dammam slaughterhouse, in KSA and identified based on 16S rRNA gene sequencing. Phylogenetic analysis revealed its closeness to *Bacillus mojavensis*. In comparison with static culture, shake culture recorded 27.9-fold increase. FT-IR Spectroscopy showed sharp bands at 3415.6, 2942.1, 1646.5, 1183.1 and 1111.8 cm^{-1} that are typical for carbohydrate. Furthermore, strong absorbance in 1200-950 cm^{-1} indicated polysaccharide nature of the polymer. Result of GPC indicated that weight average (Mw), number average (Mn) and size average molecular weight (Mz) for EPS polymer were 1151Da, 987Da and 1302Da, respectively. Maximum EPS yield (5.62 mg/mg) was reported during growth on M3 medium supplemented with C:N ratio of 4:1 for sucrose and ammonium sulfate, respectively. Supplementation of medium with trace element solution-I resulted in remarkable decrease in EPS yield (1.62 mg/mg). Maximum EPS production (63.02 mg/mg) was recorded during growth on synthetic medium M3 in presence of 20 g/L sucrose. Successful use of the agro-industrial carbon source date syrup or DEPS rather than sucrose might significantly lower process economy and increase promises for production of EPS on industrial scale.

Keywords: Exopolysaccharide; *Bacillus mojavensis*; FT-IR spectroscopy; GPC molecular weight; EPS production.

Exopolysaccharides are naturally synthesized macromolecules containing repeated units of sugar moieties, secreted into the medium during growth of many organisms including bacteria^{1, 2}. In many bacterial species, EPSs are involved in bacterial biofilm formation³. Bacterial exopolysaccharides (EPS) gains tremendous imputes in numerous health and industrial applications namely as; antitumor, antioxidants, anti-inflammatory, bioabsorbants, bioflocculants, encapsulation materials, drug carriers, ion exchange resins and emulsifying agents due to their unique physical and chemical properties^{1, 4-8}. Production of EPS by bacteria belong to genus *Bacillus*, was

reported by many scientists⁹⁻¹². Although many exopolysaccharides are currently available in market including; alginate, xanthan, carrageenan, galactan, leuvan, cellulose and hyaluronic acid, the interest in exploitation of valuable EPS for industrial applications has been increased¹³⁻¹⁶.

In this study an attempt was made to isolate, characterize and identify an EPS producing bacilli from soil, sewage and air samples from Dammam city, Eastern province, Saudi Arabia. Potent strain was identified by 16S rDNA analysis revealing its closeness to *B. mojavensis*. Chemical characterization of the produced polymer was analyzed by FT-IR spectroscopy and GPC techniques to detect the distinctive peaks of the polymer. Emphasis was given to the impact of different media composition on EPS production as well as the use of the raw agro-industrial waste

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(DEPS) as a sole C source during EPS production from *B. mojavensis* DAS10-1.

MATERIALS AND METHODS

Microorganism; isolation and identification

Screening was carried out among a group of bacilli isolated from different sources in Dammam city, Eastern Province, Saudi Arabia as previously reported (17-18). Bacterial candidates were tested for exopolymer production by cultivation on complex medium of the following composition (g/L): Beef; 3, peptone, 5; K_2HPO_4 ; 3, NaCl; 5 with agar; 15 as a solidifying agent and supplemented with 10 g/L glucose (M1) or sucrose (M2) as a carbon source. Visible viscous colonies were selected and the most potent EPSs producing were chosen for further investigation. Isolates maintained on nutrient agar slant composed of (g/L): peptone; 5, beef extract; 3, NaCl; 2 and agar; 20. Stock cultures were subcultured at regular intervals of one month and stored under refrigeration.

Molecular identification by 16S RNA gene analysis

Potent EPS producing strain *Bacillus* sp DAS10-1 was identified by amplification of 16S RNA gene sequencing using set of primers previously described in Berekaa *et al.* (18). After PCR amplification and subsequent sequencing 16S rDNA sequence was aligned with published sequences through BLAST sequence tool from NCBI database. Subsequently, sequence deposited in the GenBank under the accession number KU199819. The other six bacilli strains were molecularly identified and accession numbers were given as previously reported¹⁸.

Growth and production conditions

The bacterium grew in 50 ml aliquot of nutrient broth dispensed in 250 ml Erlenmeyer flask and incubated at 37°C for 24 h at 120 rpm as a seed culture. For quantitative estimation of EPS 1.5% inoculum of overnight culture was used to inoculate complex media M1 and M2 with glucose or sucrose as a sole carbon source, respectively. For optimization of EPS production another 5 modified basal salt production media (M3-M7) were tested. M3 basal production medium with the following composition (g/L): Sucrose, 20; K_2HPO_4 ; 8, KH_2PO_4 ; 2, $MgSO_4 \cdot 7H_2O$; 0.5; $(NH_4)_2SO_4$; 5

and traces of yeast extract. M4 was similar to M3 medium plus 1.5 mL trace element solution-I with the following composition; $FeSO_4 \cdot 4H_2O$, $CaCl_2 \cdot 2H_2O$, $MnSO_4 \cdot 4H_2O$, $ZnCl_2$, 1 mM each). However, M5 was modified M4 medium with equal amount of sucrose and ammonium sulfate (10 g/L). Furthermore, M6 was similar to M3 medium plus 1.5 mL trace element solution-II with the following composition (g/L): ($FeSO_4 \cdot 7H_2O$, 10; $CaCl_2$, 2; $MnSO_4 \cdot 4H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 2.2; $CuSO_4 \cdot 5H_2O$, 1.9; $(NH_4)_2Mo_7O_{24}$, 0.1; $Na_2B_4O_7 \cdot 10H_2O$, 0.02). Finally, M7 medium was similar to M6 plus equal concentration of sucrose and ammonium sulfate (10 g/L). The most potent exopolymer-producing bacteria were cultivated in 50 mL aliquot of the tested medium and dispensed in 250 mL Erlenmeyer flask and incubated at 37°C for 48 h under shake condition.

Extraction and quantification of EPS

For recovery of exopolysaccharide biopolymer, modified method of Kumar *et al.*¹⁹ was applied. Cultivation medium was first centrifuged to remove cells (30 min, 5000 rpm at 4°C) and the EPS was precipitated using ice-cold ethanol (1:2 volume ratio) and kept at 4°C overnight. Crude precipitated EPS was separated by centrifugation and purified by repeated precipitation for 2 times. Finally, precipitate was dried overnight at 60°C and weighed. For estimation of cell dry weight, harvested cells washed once with distilled water, dried and weighed till constant weight.

Characterization of EPS polymer by FT-IR spectroscopy

Chemical characterization of exopolysaccharide biopolymer was carried out by Fourier transform infrared spectroscopy (FT-IR). Extracted dry EPS polymer from *B. mojavensis* DAS10-1 was used in FT-IR analysis. Spectra were recorded between 400 and 4000 cm^{-1} using Nicolet 6700 FITR spectrometer from the Nicolet Instrument Corporation, USA.

Determination of EPS molecular weight by GPC

Gel Permeation Chromatography (GPC) instrument from Waters Corporation (USA) equipped with refractive index detector (RI) model 2410 and HPLC pump 515 was applied to identify each of weight average molecular weight (Mw), number average molecular weight (Mn) and size average molecular weight (Mz) for the EPS polymer. Practically, 200 μ L of the extracted EPS

polymer dissolved in THF was injected to Waters Styragel column and then isocratically eluted with THF as a mobile phase. The Flow rate of the mobile phase was adapted to be 1 mL/min, while, the temperature of column was 40°C. The calibration was done using polystyrene standards and the data managed by GPC data processing software.

RESULTS

Isolation, Identification of EPS producing bacteria

In a screening program for exploring microbial EPS production, seven bacterial strains belong to genus *Bacillus* sp were detected. The strains showed highly viscous growth on solid basal medium supplemented with glucose or sucrose as a carbon source. Results of polymer productivity (Table 1) indicated that both complex media M1 and M2 support EPS production by *Bacillus* strains, in different degrees. Among tested strains, *Bacillus* sp. DAS10-1 and *Bacillus megaterium* DG8 showed the optimal EPS productivity especially on M2 medium supplemented with sucrose (8.54 and 6.89 mg/mg, respectively). Morphologically, bacterial strain *Bacillus* sp DAS10-1 was rod in shape, Gram positive and spore forming. It is capable of growing on different sugars such as: sucrose, glucose, fructose, mannose, xylose and arabinose. It showed catalase, amylase and protease enzymatic activities. It is also sensitive to wide number of antibiotics including; ampicillin (10 µg), tetracycline (30 µg), erythromycin (5 µg), kanamycin (30 µg), amoxicillin (30 µg) and neomycin (30 µg) however, less sensitive to streptomycin (10 µg). Additionally, it showed high sensitivity to toxic metals such as Cr (2 mg/mL); Ag, Zn (4 mg/mL) and Hg (1 mg/mL) ions while, showed high tolerance to Pb (4 mg/mL). It showed alpha-hemolytic activity during growth on blood agar medium. For phylogenetic analysis, complete 16S rRNA gene of the most potent EPS producing strains was amplified and partially sequenced. Analysis of *Bacillus* sp. DAS10-1 16S rDNA gene sequence showed high similarity to the corresponding sequences of *Bacillus mojavensis* strains deposited in GenBank, NCBI, NIH, USA.

FT-IR spectroscopy analysis

For chemical characterization, *B. mojavensis* DAS10-1 cells were cultivated on

liquid production medium M3 and EPS polymer was extracted. FT-IR Spectroscopic analysis of purified EPS polymer (Figure 1) showed clear bands at 3415.62, 2942.13, 1646.51, 1183.01 and 1111.81 cm⁻¹. Additionally, strong absorbance in the region 1200-950 cm⁻¹ was observed.

Molecular weight of EPS produced by *B. mojavensis* DAS10-1

Under the above mentioned separation conditions (in the experiment part) only one peak was noticed and identified at retention time of 34.2 min. in the GPC chromatogram shown in figure 2. The identified polymer was of weight average molecular weight (Mw), number average molecular weight (Mn) and size average molecular weight (Mz) 1151Da, 987Da and 1302Da, respectively.

Production of EPS biopolymer by *B. mojavensis* DAS10-1

For further analysis, *B. mojavensis* DAS10-1 cells were cultivated on complex M2 medium and incubated under different aeration conditions. Results indicated that polymer production is dependent on aeration condition. Maximum EPS yield (16.2 mg/mg) was recorded when cells were incubated at 120 rpm (approximately 27.9-fold increase polymer yield in comparison with the static culture).

Exopolysaccharide (EPS) production on different media

Level of two main critical parameters involved in EPS production were simultaneously tested namely; C:N ratio and the use of trace element solution (-I & -II). Results in figure 3 revealed that maximum EPS yield (5.62 mg/mg) was recorded during growth on M3 medium with 4:1 C:N ratio, without trace element solution. Furthermore, addition of any of these trace-element solutions (-I or -II) to modified basal production media resulted in dramatic decreased in EPS yield, even in presence of equal or 4:1 C:N ratios (M4-M7). Interestingly, the EPS produced during cultivation on complex medium (M2) recorded approximately 2.89-fold increase in yield. Results also indicated that EPS production enhanced by increasing sucrose concentration as well as trace amount of yeast extract.

Production of EPS by *Bacillus mojavensis* DAS10-1 using different sucrose levels

Results in figure 4 indicated that EPS gradually increased with sucrose concentration

increase while, the maximum EPS production (63.02 mg) and yield (5.48 mg/mg) were noticed in presence of sucrose (20 g/L). Further increase in sucrose concentration led to remarkable increase in cell biomass while, lowering EPS yield.

Production of EPS by *Bacillus mojavensis* DAS10-1 using DEPS

Production of EPS by *B. mojavensis* DAS10-1 during growth of bacterial cells on date

syrup “DEPS”; most dominant raw feedstock material in Saudi Arabia, was tested. Bacterial cells were cultivated on M3 medium in presence of DEPS as a sole carbon source. Results revealed successful production of EPS polymer in presence of DEPS as a carbon source. Surprisingly, increasing DEPS concentration from 0.5 to 1 mg/L led to simultaneous increase in polymer yield from 5.6 to 6.93 (mg/mg), respectively.

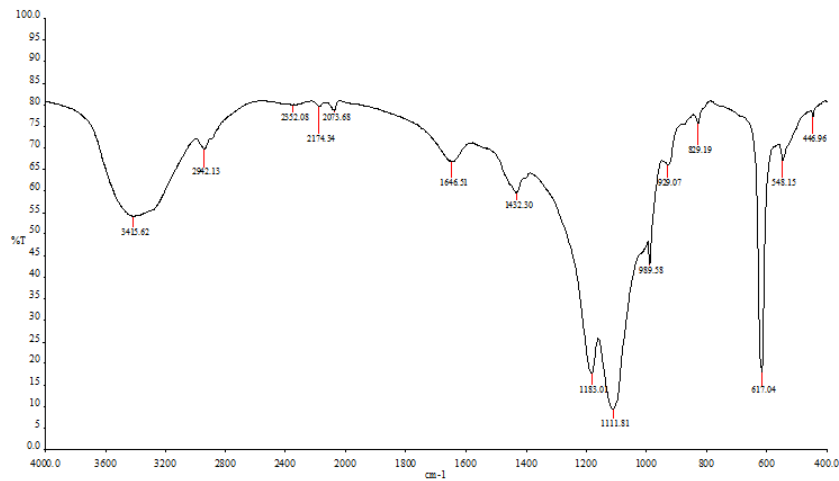


Fig. 1. FT-IR spectroscopy of exopolysaccharide (EPS) polymer produced from *B. mojavensis* DAS10-1

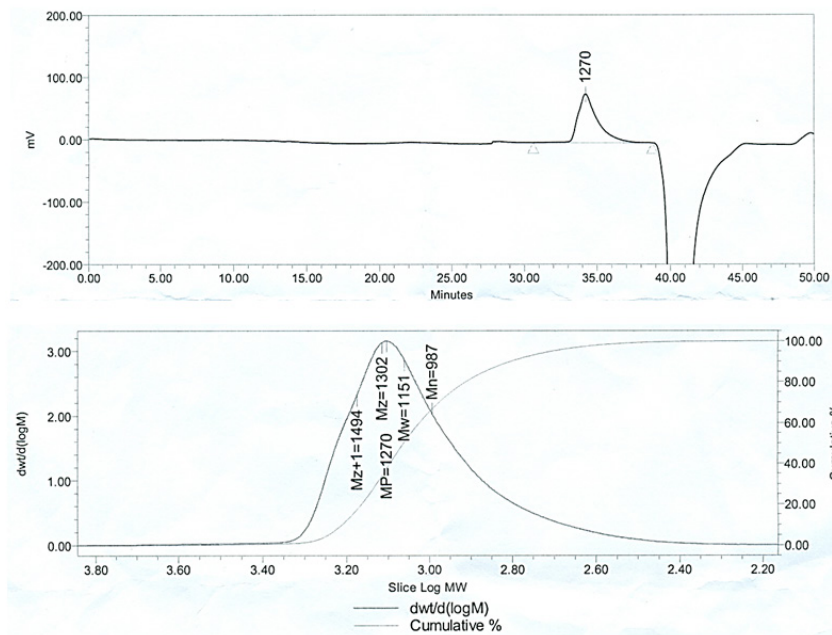


Fig. 2. Gel permeation chromatogram and Molar Mass Distribution pattern of exopolysaccharide (EPS) polymer produced by *B. mojavensis* DAS10-1

DISCUSSION

Bacterial exopolysaccharides (EPS) gains tremendous impetus in numerous health and industrial applications due to their unique physical and chemical properties⁶⁻⁸. In this study, group of bacilli explored for potential EPS production (Table 1). Among tested strains, *Bacillus* sp. DAS10-1 showed the optimal EPS productivity. 16S rDNA gene was partially sequenced. This sequence deposited in the GenBank with the accession number KU199819. On the other hand, the other 6 bacilli were previously identified by 16S rDNA

gene analysis and accession numbers were given as reported in Berekaa *et al*¹⁸. Analysis of *Bacillus* sp. DAS10-1 16S rDNA gene sequence showed high similarity to the corresponding sequences of *Bacillus mojavensis* strains. Sequence recorded close similarity to xylanase producing bacterium *B. mojavensis* (accession No. KC297104), endophytic bacteria *B. mojavensis* (accession No. KY127359 and KY127359), and *B. subtilis* strains involved in phosphate solubilization (accession No. KX710213) and mannanase production (accession No. FJ485822).

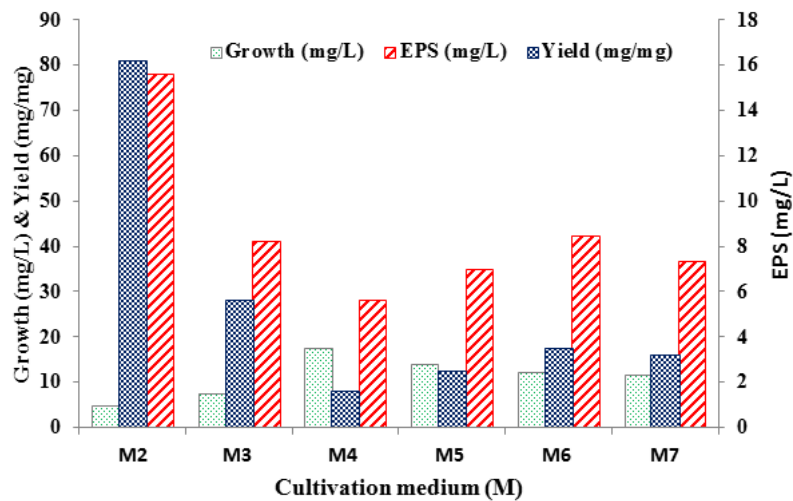


Fig. 3. EPS production by *B. mojavensis* DAS10-1 cells cultivated on different production media

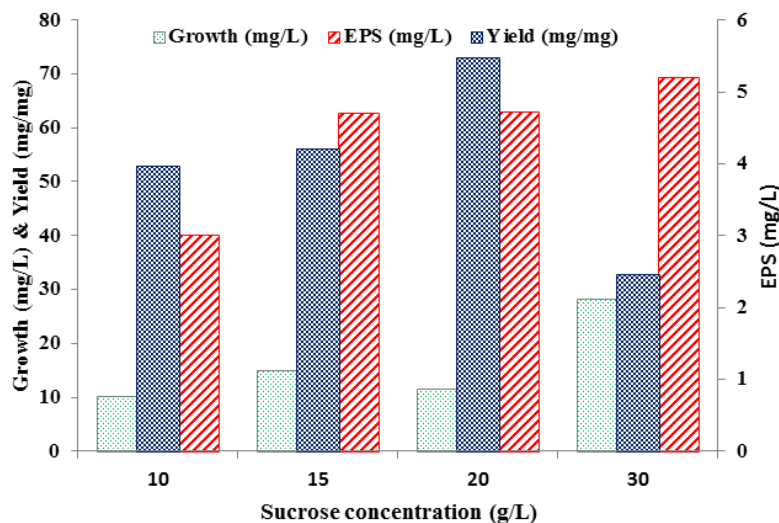


Fig. 4. EPS production by *B. mojavensis* DAS10-1 cells cultivated in presence of different sucrose concentration

Table 1. Screening of different bacteria belong to genus *Bacillus* sp for exopolysaccharide production

Bacterial Strain	EPS Yield (mg/mg)	
	M1 (Glucose)	M2 (Sucrose)
<i>B. megaterium</i> DT7 (KU199811)	4.23	3.33
<i>B. megaterium</i> DPS6 (KU199807)	4.51	3.75
<i>B. megaterium</i> DPS8 (KU199804)	3.74	2.56
<i>B. megaterium</i> DG8 (KU199809)	5.45	6.89
<i>B. megaterium</i> DP7 (KU199812)	4.63	1.94
<i>B. axarquiensis</i> DSG5 (KU199808)	3.87	4.24
<i>B. mojavensis</i> DAS10-1 (KU199819)	6.69	8.54

FT-IR Spectroscopic analysis of purified EPS polymer showed clear band at 3415.62 cm^{-1} due to -OH stretching or hydroxyl stretching vibration of the polysaccharide²⁰⁻²². Bands due to C-H stretching vibration (C-H asymmetric) in the sugar ring and C-O and carboxyl group stretching vibration were identified at 2942.13 cm^{-1} and 1646.51 cm^{-1} peaks, respectively^{20, 22}. The bands at 1183.01 cm^{-1} and 1111.81 cm^{-1} due to valent vibration of C-O-C bond and C-O bond, respectively are typical for carbohydrate^{20, 21, 23}. In concordance with Kavitatek *et al*¹⁴, the presence of strong absorbance in the region 1200-950 cm^{-1} indicated the polysaccharide nature of the EPS. The FT-IR pattern collectively showed very close similarity with the results obtained by Singh *et al*²⁴ during profiling of EPS produced by *B. licheniformis* associated with seaweeds. GPC is known to be one of the powerful tools to separate the different type of polymers according to their molecular size²⁵. Interestingly, only one polymer species was recorded. The molecular values of the identified polymer Mw, Mn and Mz 1151Da, 987Da and 1302Da, respectively were characteristics for polymer. However, the polydispersity value (PDI) was 1.16 (very close to 1) indicating only one length of polymer is present which usually belongs to biological polymers²⁶.

In the current study, production of the EPS polymer was enhanced by increase in aeration condition. Likewise, the increase of aeration and rate of agitation resulted in enriched exopolysaccharide production by *Aeribacillus palidus*. This is due to the influence of aeration on availability of nutrient, dissolved oxygen as

well as the rate of metabolite release from cells²⁷. Also, it was evident that 4:1 C:N ratio is suitable for optimal EPS yield. Ratio of carbon to nitrogen plays a crucial role in microbial biomass as well as exopolymer production with favor to higher carbon to decreased nitrogen content as reported by Abdul Razack *et al*²⁸. Moreover, EPS production was enhanced by increasing sucrose concentration and with traces of yeast extract. It is assumed that the disaccharide sugar, sucrose could act as a precursor of EPS in many bacteria²⁸. Production of EPS in presence of yeast extract was previously reported by many authors^{29,30}. Mostly, yeast extract was used as vitamin and nitrogen source^{30,31}.

Interestingly, *B. mojavensis* DAS10-1 showed great capability of EPS production during growth on date syrup or "DEPS"; most dominant raw feedstock material in Saudi Arabia. Production of EPS from *B. subtilis* using agro-industrial wastes such as rice bran and sugar can molasses was reported by Abdul Razack *et al*²⁸. Likewise, date syrup was successfully used as a raw material for bacterial production of levan³². It is assumed that sugars components in date syrup "DEPs" (mainly sucrose) can be used as a carbon source by many microbes during biopolymers production^{32, 33}.

CONCLUSION

In conclusion, *B. mojavensis* DAS10-1 is recognized as a potent EPS producer. FT-IR spectroscopy and GPC analysis revealed characteristics typically for polysaccharides. Maximum EPS yield was recorded with C: N ratio of 4:1 for sucrose and ammonium sulfate.

Raw agro-industrial waste (DEPS) can be used as alternative substrates for commercial production of EPS.

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