In vitro Exopolysaccharide Production of Some Soil Bacteria Isolated from Soil Affected by Salt

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ABSTRACT

In this study, production of exopolysaccharide (EPS) from soil bacteria isolated from the salinity soil and the effects of agitation, some carbon sources such as glucose, fructose, xylose, rhamnose and NaCl levels the on production of polysaccharide playing a role in soil aggregation were studied. Maximum EPS production for *Acinetobacter* sp., *Pseudomonas fluorescens* and *Sphingomonas paucimobilis* was obtained 72 h of incubation and maximum EPS production for *Bacillus simplex* was obtained at 96 h. Maximum EPS production was obtained at 150 rpm for all bacteria studied. Glucose gave the best result for all of bacteria. *Bacillus simplex* produced higher EPS and viscosity than the other bacteria. NaCl levels affected both the growth and exopolysaccharide production of the bacteria. By promoting aggregate formation, EPS produced bacteria such as *S.paucimobilis*, *B.simplex*, *Acinetobacter* sp. and *P.fluorescens* may have created favorable growth.

Keywords: Agitation, carbon source, exopolysaccharide, soil bacteria, NaCl

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I. INTRODUCTION

Exopolysaccharides produced by bacteria are a complex biofilm of high molecular polymers [1]-[3]. Exopolysaccharides (EPS) are involved in the formation of microbial aggregates, bioremediation, plant-microbe interaction and adhesion to surface [4], [5]. The production of EPSs by bacteria causes sand particle to adhere and build up an aggregate. EPS can protect the cells from various stressess such as temperature, NaCl, drought, pH and provide with energy and carbon source in decreasing nutrient limiting conditions [6], [7]. The efficiency in conducting forced aeration is linked to agitation in culture. The carbon source plays a major role in the synthesis of polysaccharide by microorganisms [8].

Carbohydrates are an important source of nutrients for microorganisms. In order to identify a suitable carbon source for exopolysaccharide production by tested bacteria; different carbohydrates, i.e. glucose, fructose, xylose, rhamnose were used. Many bacteria such as *Sphingomonas*, *Rhizobium*, *Pseudomonas*, *Bacillus* and *Streptococcus* produces EPS. Also, *Azotobacter vinelandii*, *A.chroococcum*, *Acetobacter xylinum*, *Burkholderia caribensis*, *Azospirillum* have also been reported to produce EPS [7], [9], [10]. Some isolates of *Bacillus* species have been known to produce EPS such as levan, mannan and acidic polysaccharides [11]. EPSs have been largely studied for either understanding their role in the environment [12]-[14] or assessing their potential applications in the industrial applications [15]-[17].

The influence of EPS by producing rhizobacteria on the aggregation of root adhering soils was demonstrated by Ashraf *et al.* [18]. It has been reported that EPS produced by soil bacteria is effective in the formation of rhizospheric soil aggregation and in determining the volume of macroporous soil [2]. Chenu and Guerif [19] reported that exopolysaccharides produced from bacteria are the molecules that increase the plasticity of clay and increased the mechanical strength of clay minerals. It has been reported that EPS produced by bacteria in the soil plays an important role in collecting soil around the roots and in the structural stability of the salt in the soil [4,18]. Harran Plain is one of the most important agricultural regions of Turkey and has a great potential for the major agricultural products of the Southeastern Anatolian in Turkey [20].

Harran Plain is important in Turkish agricultural production. Harran Plain has 225 000 ha area and currently 132 000 ha area is now irrigated. 15 000 ha area have saline and sodicity problems due to excessive irrigation and poor drainage [20]. EPS production specifically important mechanism in salt affected soils. With regard to bacteria isolated from saline soil in Turkey, there is not such work. In this study, the effects of different carbon sources, NaCl and aeration on the exopolysaccharide production of bacterial isolates from soil affected by salt were investigated.

II. MATERIALS AND METHODS

A. Study Site and Soil Sampling

The bacteria used in this study were isolated from soil affected by salt in the plain of Harran in Turkey. Some physiochemical characteristics of used soil were shown in Table 1. The soil management is irrigated. The soil samples were taken from cotton cultivated areas. Physical and chemical properties of the soil used in this study were determined in samples taken from 0 to 15 cm depth.

B. Routine Soil Analysis

After air-dried soil samples were sieved through a 2 mm sieve. The soil pH measured by pH meter, electrical conductivity measured by EC meter [21]. CaCO_3 contents were determined by volumetric method [22]. Organic matter contents were determined by the wet oxidation method with $\text{K}_2\text{Cr}_2\text{O}_7$ [23]. Texture, total nitrogen, K and Na contents were determined according to Martin and Reeve [22]. Some characteristics of the soil are given in Table I.

TABLE I: PHYSICAL AND CHEMICAL PROPERTIES OF THE SOIL USED

Property	Value
Particle size distribution	
Sand (%)	22.88
Silt (%)	24
Clay (%)	53.12
Texture grade	Clay
pH	7.74
EC (dS m^{-1} at 25 °C)	2.45
Total N (%)	0.18
Available K (kg/da)	97.2
CaCO_3	21.6
Organic matter (%)	1.14
Na^+ (me/100 g)	2.06

C. Bacterial Identification

The bacteria isolated were identified as described by Ashraf *et al.* [6]. Briefly, the isolates were classified into Gram negative and Gram positive according to Gram staining and identified by their fermentation pattern in the VITEC system (bioMerieux) using a VITEC biochemical cards.

D. Culture Conditions for Exopolysaccharide Production

The RCV medium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g l^{-1} ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g l^{-1} ; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.022 g l^{-1} ; EDTA, 0.02 g l^{-1} ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.43 mg l^{-1} ; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.30 mg l^{-1} ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.75 mg l^{-1} ; H_3BO_3 , 2.80 mg l^{-1} ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 26 $\mu\text{g l}^{-1}$; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 70 $\mu\text{g l}^{-1}$; K_2HPO_4 , 5.2 mM; KH_2PO_4 , 4.4 mM; pH 6.8) was used to determine the effects of carbon sources on bacterial EPS production [24]. Bacteria were grown (10^8 cfu ml^{-1}) in RCV mineral medium containing 10 g l^{-1} of either glucose, fructose, xylose and rhamnose at 28 ± 2 °C. Carbon sources were separately sterilized by filter and added to the cooled basal media.

E. Viscosity Measurement

Viscometer (NDJ-1 model, Soft optical instruments) was used to determine the viscosity in the growth media. The pH of the samples was determined using a pH meter.

F. Effect of Agitation on EPS Production of Cells

Modifications of agitation (50, 100, 150 and 200 rpm) were also made to study the influence of culture conditions on exopolysaccharide production.

G. Effect of NaCl Levels on EPS Production of Cells

The cultures were inoculated into RCV medium containing different concentrations of NaCl (0, 50, 100 and 150 mM NaCl (w/v)) and incubated at 150 rpm, 28 °C. Optical density was measurement at 540 nm by spectrophotometer. The experiments was conducted in three replicates.

H. Exopolysaccharide Determination

After inoculation, broth cultures were incubated at 28 °C for 24 h. Bacterial culture is boiled for 15 min at 100 °C, cooled and TCA was added. The contents were vortexed, centrifuged for 30 min at 9000 rpm 4 °C. The ethanol was added to the supernatant, the supernatant was stored at 4 °C for 1 night, the content was centrifuged. The pellet was dissolved in pure sterile water and the phenol sulfuric acid method was applied [25]. To determine the amount of EPS, a standard curve was prepared using the phenol sulfuric acid method using glucose. The EPS amount was calculated using the standard curve [8].

I. Statistical Analysis

The results were statistically analysed by the JMP11, the treatments means were compared by Tukey's test ($p \leq 0.05$) and studied triplicated replications.

III. RESULTS AND DISCUSSION

Acinetobacter sp., *Bacillus simplex*, *Sphingomonas paucimobilis*, *Pseudomonas fluorescens* were selected due to their high number in soil. They were identified as the Gram negative bacteria *Acinetobacter* sp., *Sphingomonas paucimobilis*, *Pseudomonas fluorescens* and the Gram positive bacteria *Bacillus simplex*. Databases using VITEC program, confirmed the isolates belonged to the *Acinetobacter* sp., *Bacillus simplex*, *Sphingomonas paucimobilis*, *Pseudomonas fluorescens* (Table II). All bacterial strains grow fast medium supplemented with 100 mM NaCl (Table II).

Four strains secrete the enzymes protease and catalase. EPS production by *Acinetobacter* sp., *B. simplex*, *S. paucimobilis*, *P. fluorescens* were investigated for different carbon sources, incubation times (24, 48, 72, 96, 120 h) and agitation (50, 100, 150, 200 rpm) in pure culture medium. Preliminary incubation experiments were performed in order to determine the effect of incubation period on EPS by the bacteria. Samples were removed at intervals (24, 48, 72, 96 and 120 h) and quantitative extraction of EPS was carried out. According to these results, maximum EPS recovery for *Acinetobacter* sp., *P. fluorescens*, *S. paucimobilis* was 312.4, 410.6 and 345.8 $\mu\text{g ml}^{-1}$, respectively. After of 72 h of incubation, EPS production by *Acinetobacter* sp., *P. fluorescens* and *S. paucimobilis* started to decrease. Maximum EPS recovery for *B. simplex* (418.2 $\mu\text{g ml}^{-1}$) was obtained at 96 h, and therefore were selected to evaluate EPS production in further experiments (Fig. 1).

TABLE II: IDENTIFICATION AND SOME PROPERTIES OF BACTERIAL STRAINS ISOLATED FROM SALT AFFECTED SOIL

Isolate no	Identified as	% similarity	Salt tolerance *	Capacity for at 37 °C
BHO1	<i>Bacillus simplex</i>	99	+	+
BHO62	<i>Acinetobacter</i> sp	99	+	+
BHO45	<i>Sphingomonas paucimobilis</i>	99	+	+
BHO78	<i>Pseudomonas fluorescens</i>	99	+	+

*highest salt concentration (100 mM) which didn't affect growth

Fig. 1. Effect of incubation times on EPS production of tested bacteria. Error bars indicated Standard deviation of three parallel ($p \leq 0.05$).

Results for growth and EPS production of tested bacteria are shown in Table III. The growth and EPS productions of bacteria were affected by carbon sources (Table III). Carbon source is required for growth and EPS production of bacteria [26].

TABLE III: INFLUENCE OF CARBON SOURCES ON EPS PRODUCTION

Bacteria	Carbon Sources	Viscosity (mPas)	pH	EPS (µg/ml)
<i>Acinetobacter</i> sp.	Glucose	2.09	7.48	817.2±0
	Fructose	1.78	6.11	541.0±2
	Xylose	1.98	6.23	775.6±1
	Rhamnose	1.83	5.47	586.1±2
	Control	1.60	6.92	421.0±1
<i>B. simplex</i>	Glucose	2.70	6.06	1204±2
	Fructose	2.22	7.08	688.4±1
	Xylose	2.36	6.24	987.0±0
	Rhamnose	2.40	5.66	1176±0
	Control	2.10	7.01	568±0
<i>S. paucimobilis</i>	Glucose	2.35	7.04	951±1
	Fructose	2.21	7.81	743±1
	Xylose	2.32	6.21	652±2
	Rhamnose	2.28	6.02	874.2±1
	Control	2.11	6.83	371±1
<i>P. fluorescens</i>	Glucose	2.02	7.04	824.2±1
	Fructose	1.83	7.81	811.2±0
	Xylose	2.17	6.21	745±2
	Rhamnose	2.00	6.02	692.8±2
	Control	1.78	6.46	386±0

*Values are the means ± standard deviations of triplicate measurements

Acinetobacter sp. has produced 817.2 µg ml⁻¹, 775.6 µg ml⁻¹, 586.1 µg ml⁻¹ and 541 µg ml⁻¹ exopolysaccharide

respectively in media containing glucose, xylose, rhamnose and fructose, respectively. In *Bacillus simplex*, maximum EPS amount at glucose was 1204 µg ml⁻¹ (Table III). In *P. fluorescens*, maximum EPS amount at glucose and fructose were 824.2 µg ml⁻¹ and 811.2 µg ml⁻¹, respectively. In *S. paucimobilis*, maximum EPS amounts at glucose, rhamnose, fructose and xylose were 951 µg ml⁻¹, 874.2 µg ml⁻¹, 743 µg ml⁻¹ and 652 µg ml⁻¹, respectively (Table III). EPS production may have been affected due to the nature of the carbon sources used. The results demonstrate that *Acinetobacter* sp., *B. simplex*, *P. fluorescens* and *S. paucimobilis* are capable of utilizing various sugars for EPS production. Glucose gave the best result for all of bacteria (Table III). Thus whether a sufficient supply of carbon sources was efficient for a EPS production seems to be dependent on the characteristics of the bacteria. Different microorganisms are reported to produce variety of exopolysaccharide [1], [2], [11], [27].

Tewari and Arora [7] reported about the utilization of glucose by *Pseudomonas aeruginosa* for EPS production. For the *Rhizobium hedysarum* isolate, glucose and sucrose were the most suitable source for EPS production [28]. The effect of agitation on viscosity and EPS production was observed in medium containing glucose (Fig. 2). Breedveld *et al.* [29] reported that the growth of microorganisms and increases in viscosities were dependent on aeration of the media. The growth of all bacteria and EPS production increased with the increase in agitation. However, the best EPS production was found at 150 rpm for all bacteria (Fig 2). It has been reported that the *Rhizobium* isolate promotes polysaccharide synthesis when mannitol and sodium nitrate are added to the culture medium by forced agitation [8]. Several bacteria are known to synthesis large amounts of extracellular polysaccharides which resulting in mucoid colonies on agar plates and extremely viscous broth culture media [15], [30], [31]. In this study, the increase in agitation increased the viscosity (Fig. 2). These results are similar to those of other researchers [8], [32], [33].

Fig. 2. Effect of agitation on viscosity of tested bacteria.

Tewari and Arora [7] found that the increase in EPS produced by *Pseudomonas* isolate was effective in protecting the isolate in soil. The production of EPS in bacteria is influenced by various environmental parameters such as temperature, salinity, pH [26]. The growth (Fig. 3) and EPS production of bacterial isolates were influenced by NaCl levels. Thus, EPS produced by bacteria as osmoprotective agent may protect bacteria from osmotic stress.

Increase in EPS production in saline medium suggests that under stress condition energy flow of tested bacteria may be directed toward protective mechanism. Similar results have been examined in other studies [2], [34]. Maximum growth by these bacterial isolates was recorded with 100 mM NaCl. NaCl concentrations (50 mM and 100 mM) in glucose containing medium have stimulated growth however, at concentration of 150 mM NaCl, bacterial growths have decreased (Fig. 3). Salinity is a significant factor influencing the physiology of a microorganisms by affected nutrient uptake, enzyme activity [35]. In order to improve EPS production by bacterial isolates, the influence of NaCl was studied. The results of EPS production and growth were given in Table 4. As shown in Table 4, EPS amounts produced by bacteria were higher than their controls. Also a maximum EPS productions of tested bacteria were recorded with glucose and NaCl.

Fig. 3. Influence of NaCl levels on bacteria grown.

Geddie and Sutherland [12] reported that salinity alleviation due to EPS produced by bacteria may be attributed to Na^+ removal from the medium due to biosorption. Kang and Park [10] have been reported that the EPS produced by *Acinetobacter* sp. was increased with increase of NaCl concentration. The increase in the amount of salt was limited the increase in EPS production. In stress tolerating *Bacillus simplex* there was increase in EPS production by about, 139.2, 123.8, 117.1 and 68.1 % according to control, at glucose + NaCl (100 mM), fructose + NaCl (100 mM), xylose + NaCl (100 mM) and rhamnose + NaCl (100 mM), respectively (Table IV). Exopolysaccharides include molecules released by microorganisms in their nature environment and laboratory conditions [9,26,30].

TABLE IV: INFLUENCE OF NAACL (100 MM) ON EPS PRODUCTION

Bacteria	Media	O.D.* (540 nm)	EPS *(µg/ml)
<i>Acinetobacter</i> sp	Glucose + NaCl	2.47	292±1
	Fructose + NaCl	2.17	265±0
	Xylose + NaCl	2.23	194±0
	Rhamnose + NaCl	1.96	204±1
	Control + NaCl	1.82	158±2
<i>B. simplex</i>	Glucose + NaCl	2.54	421±0
	Fructose + NaCl	2.23	382±0
	Xylose + NaCl	2.18	292±1
	Rhamnose + NaCl	2.43	394±1
	Control + NaCl	2.09	176±1
	Glucose + NaCl	2.01	402±0
	Fructose + NaCl	1.93	345±1

<i>S. paucimobilis</i>	Xylose + NaCl	1.65	366±2
	Rhamnose + NaCl	1.98	213±2
	Control + NaCl	1.70	152±2
<i>P. fluorescens</i>	Glucose + NaCl	2.62	384±0
	Fructose + NaCl	2.25	327±0
	Xylose + NaCl	2.10	286±1
	Rhamnose + NaCl	2.04	184±1
	Control + NaCl	1.67	165±1

*Values are the means ± standard deviations of triplicate measurements

The EPS produced by *Bacillus* species and *Enterobacter* species seems to play a crucial role by binding the hazardous Na^+ , thereby alleviating the adverse effects of salts on crops [2]. As EPS can protect the bacterial isolates against abiotic stress [13], [34], we were interested in establishing a relationship between their growth, viscosity and EPS production.

Increase in EPS production in glucose and NaCl containing medium suggests that under stress condition energy flow of tested bacteria may be directed toward protective mechanism. Our results were similar to those of the researchers [2], [34]. The adaptation of bacteria to environmental conditions such as salinity, temperature, pressure, heavy metals and desiccation has been reported [12], [14], [34], [36], [37]. In this study, among carbon sources; glucose had greater influence on growth and EPS production (Table V).

TABLE V: THE EFFECTS OF VISCOSITY, EPS PRODUCED BY BACTERIA

Carbon sources	Properties			
	Viscosity		Influence of NaCl	
	(mPas)	EPS (µg/ml)	O.D. (540 nm)	EPS (µg/ml)
Glucose	2.29 e	948.1 d ⁺	2.41 b	375.2 c ⁺
Fructose	2.00 b	694.9 b	2.13 b	322.3 d
Xylose	2.20 d	791.0 c	2.05 a	284.4 c
Rhamnose	2.13 c	832.3 d	2.09 b	246.4 b
Control	1.89 a	435.4 a	1.82 a	162.3 a

Bacteria	Properties			
	Viscosity		Influence of NaCl	
	(mPas)	EPS (µg/ml)	O.D. (540 nm)	EPS (µg/ml)
<i>Acinetobacter</i> sp.	1.86 a	628.9 a	2.11 b	222.8 a
<i>B. simplex</i>	2.35 c	923.3 c ⁺	2.29 c	326.7 d ⁺
<i>S. paucimobilis</i>	2.25 d	717.8 d	1.86 a	292.9 c
<i>P. fluorescens</i>	1.96 b	691.3 b	2.14 b	270.1 b

Comparing different carbon sources, fructose containing medium showed the least effects on EPS production, whereas glucose containing medium was the most efficient ($p \leq 0.05$), followed by rhamnose, xylose (Table V). Comparing different bacterial isolates, EPS production by *B. simplex* was higher than others ($p \leq 0.05$). Also, EPS production at all carbon sources with and without NaCl was higher than their controls (Table V).

The highest EPS production according to control was examined by *B. simplex* in NaCl containing medium. *B. simplex* produced EPS and viscosity than the other bacterial isolates (Table IV). When bacterial cultures were grown in media containing different carbon sources, the EPS production and viscosity increased according to control medium (Table V). The lack of water in the soil is an important environmental stresses that limit the agricultural productivity [37]. The impact of exopolysaccharide on

hydrology is a salient point and EPS can hold up to 20 times its own weight in water [36]. EPS can alter soil structure to mediate water retention hydraulic conductivity and aggregate structure [4], [36].

IV. CONCLUSIONS

In this study we examined the effects of salinity, agitation and different carbon sources on EPS produced by *Acinetobacter* sp., *Bacillus simplex*, *Spingomonas paucimobilis* and *Pseudomonas fluorescens*. Improving EPS soil produced by bacteria can be beneficial to plant growth and industry. EPS production by *Acinetobacter* sp., *P. fluorescens*, *S. paucimobilis*, *B. simplex* in culture could be beneficial to the soil bioremediation, plant growth promoting and industry. The results of this study provided the basis for the next experimental approach to investigate the exopolysaccharide production of bacterial isolates associated with soil aggregation and plant growth under saline conditions.

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