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Exopolysaccharide Production by Root Nodule Bacteria of Wild *Lathyrus cassius* Boiss.

Yabani *Lathyrus cassius* Boiss. Kök Nodül Bakterileri Tarafından Ekzopolisakkarit Üretimi

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Abstract

In this study, it was investigated that producing ability of extracellular polysaccharides (EPS) of the *Rhizobium* strains isolated from root nodules of wild *Lathyrus cassius*. The results provided that when isolates were grown in a chemically defined medium carbon and nitrogen sources itested in this study influced EPS synthesis. Maximum EPS production (1778 µg/ml and 1762.6 µg/ml, respectively) was obtained when the chemically defined medium was supplemented with mannitol and potassium nitrate, respectively which was accompanied a great increase in the production compared to the control. In all isolates, the maximum EPS production was 72 h, 150 rpm at mannitol and potassium nitrate containing medium.

Keywords: *Rhizobium* sp., isolates, exopolysaccharide, carbon and nitrogen source

Öz

Çalışmada, yabani *Lathyrus cassius*'ün kök nodüllerinden izole edilen *Rhizobium* sp. izolatlarının ekzopolisakkarit (EPS) üretimleri araştırılmıştır. İzolatlar belirli bir kimyasal ortamda geliştirildiklerinde, test edilen karbon ve azot kaynakları EPS üretimini etkilemiştir. Ortama sırasıyla mannitol ve potasyum nitrat eklendiğinde kontrolle karşılaştırıldığında EPS üretiminin (sırasıyla, 1778 µg/ml ve 1762.6 µg/ml) maksimum değerde olduğu belirlenmiştir. İzolatların tümünde maksimum EPS üretimi 150 rpm'de 72 saatte, mannitol ve potasyum nitrat içeren ortamda bulunmuştur.

Anahtar Kelimeler: *Rhizobium* sp., izolatlar, ekzopolisakkarit, karbon ve azot kaynağı

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1. Introduction

Rhizobium bacteria are able to form nodules on the root of leguminous plants and present as nitrogen fixing organelles maintained by the plant in return for ammonia (Ghosh et al. 2005). Rhizobia synthesize different classes of polysaccharide (Breedveld et al. 1993; Donot et al. 2012). *Rhizobium* species have produced EPS that are involved in plant host symbiosis (Becker and Pühler 1998).

For the cells, EPSs are thought to play a role in bacterial interactions with the defense response that could be generated in the plant host and the inimate interaction that occur between bacterial and plant cell membrans (Bomfeti et al. 2011). Also, EPS are thought to play a role in protection against desiccation, toxic compounds, bacteriophages, osmotic stress and to permit adhesion to solid surfaces and biofilm formation (Cunnigham and Munns 1984; Kaci et al. 2005). Even when different polymers can be a partially complement of structural defects in others, their size, structure, amounts and composition are determinant for their symbiotic interactions (D'Haeze et al. 2004; Oldroyd et al. 2011).

EPS production and amounts can be influenced by several factors such as composition of medium as well as incubation conditions (pH, time, temperature, osmotic stress) (Lloret et al. 1998; Ghosh et al. 2005; Rinaudi et al. 2006). EPSs produced by *Rhizobium* bacteria are the subject of an increasing number of studies (Kaci et al. 2005; Downie 2010; Sayyed et al. 2011; Janczarek et al. 2015). *Rhizobium* bacteria are soil bacteria. Rhizobial EPS are thought to play a role in determinating the host plant specificity of nodulation (Bomfeti et al. 2011). The objective of this work was to check the ability of the *Rhizobium* sp. isolated from the root nodules of wild *Lathyrus cassius* for EPS production and also to increase the production of EPS through optimization of culture conditions.

2. Materials and Methods

2.1. Isolation, identification

Bacteria were isolated from the root nodules of wild *Lathyrus cassius* according to Vincent (1970) using yeast extract mannitol agar medium. The morphological, cultural and biochemical features of the isolates were characterized by standard methods (Vincent 1970).

2.2. Culture conditions for EPS

Carbon and nitrogen source assays were carried out in basal medium (0.05% yeast extract, 1% mannitol, 0.01% $CaCl_2H_2O$, pH 7.0) (Dudman 1964; Küçük and Kıvanç 2009). Bacteria were grown (10^8 cfu/ml) in basal medium containing 10 mM of either sucrose, glucose, fructose, mannitol, rhamnose or 0.1% of either glycine, potassium nitrate, $(NH4)_2SO_4$. Carbon and nitrogen sources was separately sterilized and added to the medium later. The effect of basal medium removing mannitol and the effect of different nitrogen sources was also studied into the basal medium removing yeast extract. In order to determine the effect of the incubation period on the EPS production, isolates were grown at different incubation periods (24, 48, 72, 96 and 120 h) in basal medium.
The influence of the agitation (50, 100, 150 and 200 rpm), pH (5, 6, 7, 8 pH) and NaCl (0, 20, 50, 100 and 150 mM) on the EPS production by the isolates were also studied.

2.3. Isolation and quantification of EPS

After incubation, cultures were centrifuged for 15 min at 10 000 x g. Ammonium acetate (1M) was added to the supernatant and the EPS was precipitated by addition of 2 vol 2 propanol. The precipitate was then recovered by centrifugation and dissolved in distilled water, dialyzed against water and the retantate was concentrated by lyophilization. Yields of EPS were estimated by the phenol sulphuric acid method (DuBois et al. 1956).

3. Results and Discussion

This isolates were characterized by morphology, PHB granules, physiological and biochemical characterization (Table 1).

Table 1. Some cultural and physiological characteristics of isolates

Characteristics	Isolates				
	L1	L6	L7	L8	L11
Gram staining	-	-	-	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Mobility	+	+	+	+	+
Formation of poly-β-hydroxybutyrate granules	+	+	+	+	+
Citrate utilization	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+
Urea hydrolysis	+	+	+	+	+
Gelatine hydrolysis	-	-	-	-	-
Growth at pH					
4	-	-	-	-	-
9	+	+	+	+	+
12	-	-	-	-	-
NaCl tolerance					
2%	+	+	+	+	+
3%	+	+	+	+	+
4%					
Growth at temperature					
20 °C	+	+	+	+	+
37 °C	+	+	+	+	+
40 °C	+	+	-	+	+
42 °C	-	-	-	-	-
Acid production on YEM agar	+	+	+	+	+
Alkali production on YEM agar	-	-	-	-	-

+ positive, - negative

Results of morphological and biochemical characteristics such as Gram negative rods, motile were shown in Table 1 and tested positive for catalase and oxidase activity. Similar to these of *Rhizobium* as described (Vincent 1970). Isolates shows a mucoid phenotype on YEM agar. Preliminary experiments were performed in order to determine in the incubation time for optimum recovery of EPSs in *Rhizobium* sp. isolates. EPS production by isolates was investigated for different incubation periods (24, 48, 72, 96 and 120 h) in basal medium. Samples were removed at intervals and quantitative extraction of EPS was carried out. According to these results, maximum level of EPS production was obtained in 72 h, so this incubation period was selected for EPS production in further experiments (Fig. 1).

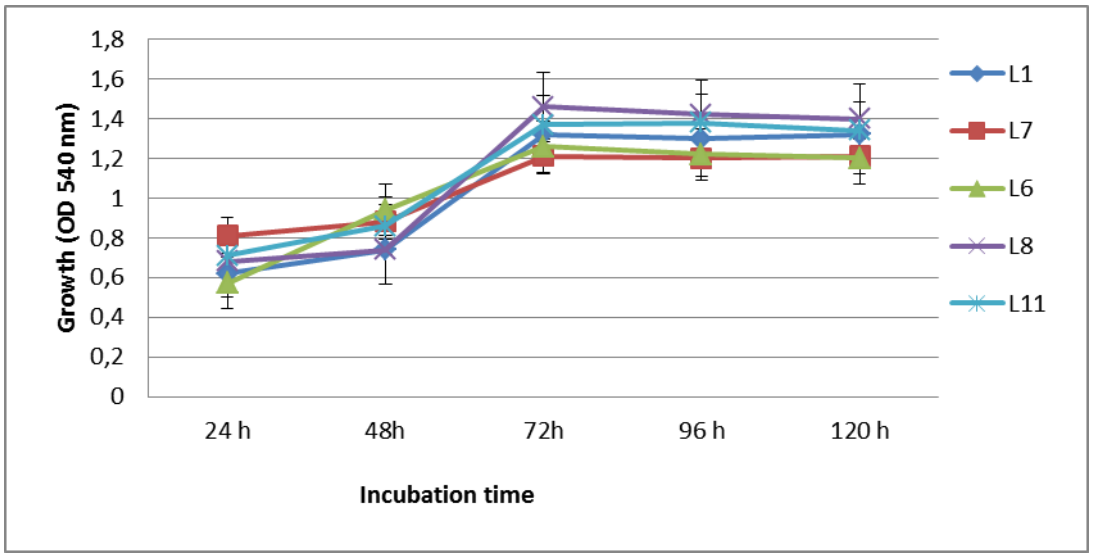

Figure 1. Effect of incubation time on IAA production of *Rhizobium* P2 in culture

In this study, we determined the effects of different carbon and nitrogen sources on growth and EPS production. Isolates were incubated in basal medium containing various carbon sources (glucose, mannitol, fructose, sucrose and rhamnose) to find a suitable carbon source for the EPS production. The results indicated in Table 2 showed that the EPS production varied from one isolate to another. Maximum OD was affected by carbon and nitrogen sources. When isolate L7 was grown in medium containing the mannitol, the EPS production (1778 µg/ml) was the highest among those tested and the control (Table 2).

The results demonstrated that isolates are capable of various sugar for exopolysaccharide production. But EPS produced from the isolates in different carbon sources were higher than their controls (Table 2). Probably, the differences could be explained on the basis of the different nature of the carbon sources employed. Data and Basu (1999), Küçük and Kıvanç (2009) reported the EPS production of *Rhizobium* bacteria using different carbon sources and the highest yield was obtained when mannitol was used as the carbon sources. Karr et al. (2000) determined that *Bradyrhizobium* was able to utilize all the tested carbon source such as glucose, mannitol, maltose to synthesize the exopolysaccharide. Hollingsworth et al. (1985) reported the EPS production of *Rhizobium* isolates using different carbon sources and the highest yield was obtained with glucose, galactose and mannose. In contrast, Janczareck et al. (2015) reported that exopolysaccharide production by isolate of *R. trifolii* in glycerol was higher other carbon sources such as sorbitol, dulcitol, arabitol, mannose, rhamnose, mannitol. Sayyed et al. (2011), reported that root nodule isolate from groundnut was able to produce the EPS in the dextrose as carbon source.

In order to improve EPS productions by isolates, the influence of nitrogen source were studied (Table 2). As described for the carbon assays, the nitrogen source affects both growth and EPS production. Potassium nitrate, ammonium sulphate and glycine showed a maximum production of 1762.6 µg/ml, 1462 µg/ml and 1020 µg/ml, respectively. Among these, mannitol and potassium nitrate had greater influence on growth and EPS production (Table 2).

However, Breedveld et al. (1993), Ghosh and Ghosh (2005) reported that mannitol and KNO_3 allowed maximum EPS production. Küçük and Kıvanç (2009) evaluated EPS production by *R. ciceri* grown in culture media with different carbon sources and found that mannitol provided the best production. In contrast, Sayyed et al. (2011) reported higher polymers production by *Rhizobium* isolate from groundnut are obtained in medium containing ammonium sulphate. Datta and Basu (1999) reported the EPS production of root nodule isolates of *Cajanus cajan* using different carbon and nitrogen sources and the highest yield was obtained when sucrose and KNO_3 were used as carbon and nitrogen sources. In this study, among tested carbon and nitrogen sources, mannitol and potassium nitrate had greater influence on EPS production. Limitation of available oxygen is known to influence growth and polysaccharide accumulation by individual organisms in very specific manner.

Table 2. Effect of different carbon and nitrogen sources on the EPS (µg/ml) production by *Rhizobium* isolates and its growth (OD at 540 nm)

Carbon sources		Isolates				
		L1	L7	L6	L8	L11
Control	OD	1.28±0.03	0.76±0.04	0.85±0.01	0.78±0.01	0.81±0.03
	EPS	304.1±0.0	188±0.1	312±0.2	291±0.1	228±0.1
Specific productivity		237.5	247.3	367.1	373.0	281.5
Sucrose	OD	1.82±0.04	1.72±0.01	1.80±0.02	1.78±0.02	1.90±0.01
	EPS	922.3±0.0	960±3.0	940±3.0	1152±1.0	970±0.0
Specific productivity[a]		506.7	558.1	522.2	647.1	392.0
Fructose	OD	1.75±0.05	1.40±0.01	1.64±0.04	1.29±0.04	1.99±0.01
	EPS	872.6±0.1	850±0.0	870±0.1	760±1.0	780±0.0
Specific productivity		498.6	607.1	530.5	589.1	787.9
Glucose	OD	1.66±0.01	1.52±0.01	1.60±0.02	1.48±0.07	1.57±0.01
	EPS	916.0±0.0	900±0.0	878±0.0	921±5.0	920±2.0
Specific productivity		551.8	592.1	548.8	622.2	585.9
Mannitol	OD	1.98±0.03	2.75±0.0	1.87±0.01	1.98±0.01	2.00±0.04
	EPS	1114±0.0	1778±0.0	1212±0.0	1405±0.0	1591±0.2
Specific productivity		562.6	646.6	648.1	709.6	795.5
Rhamnose	OD	1.92±0.02	2.00±0.00	1.92±0.04	1.95±0.05	1.86±0.02
	EPS	1023±0.1	979±0.2	1004±0.1	1002±0.0	1091±0.0
Specific productivity		532.8	489.5	523.0	513.8	586.6
Nitrogen sources		**Isolates**				
		L1	**L7**	**L6**	**L8**	**L11**
Control	OD	1.72±0.01	1.38±0.00	1.65±0.07	1.32±0.02	1.47±0.03
	EPS	522.8 ±0.2	571±0.03	472±0.0	516±0.0	566±0.0
Specific productivity		303.9	413.7	286.0	390.9	385.0
KNO_3	OD	2.75 ±0.02	2.72±0.01	2.17±0.03	2.04±0.04	3.15±0.02
	EPS	1762.6±0.1	1682±0.0	1517±0.0	1478±0.0	1704±0.1
Specific productivity		640.9	618.3	699.0	724.5	540.9
NH_4NO_3	OD	2.89±0.01	3.00±0.01	2.21±0.02	2.42±0.03	2.60±0.04
	EPS	1433.2±0.3	1438±0.0	1421±0.0	1462±0.1	1202±0.2
Specific productivity		495.9	479.3	642.9	604.1	462.3
Glycine	OD	1.80±0.05	2.04±0.05	3.17±0.05	2.72±0.02	2.94±0.06
	EPS	837.9±0.0	837.9 ±0.0	1020±0.2	924±0.2	960±0.5
Specific productivity		465.5	410.7	312.7	339.7	326.5

*Values are the means ± SD of triplicate measurements. [a]specific productivity:EPS production/growth

EPS production by *R. meliloti* (Dudman 1964) was reported to be increased significantly in low O_2 supply. Medium containing mannitol or potassium nitrate was observed in effect agitation (Fig. 2) and NaCl levels (Fig. 3) on biomass and EPS production. To ascertain the effect to NaCl on cell growth and EPS production, the cells cultivated in the mannitol or KNO_3 containing medium having different NaCl levels in a flask culture (Fig 3). EPS production by the isolates was also influenced by the NaCl levels; EPS synthesis was maximal at 50 mM NaCl (Fig. 3). In the higher NaCl levels, both cell concentration and EPS production were lowered on culture. Similar results have been reported for *R. meliloti* grown on 0.3 M NaCl (Lloret et al. 1998) and for other EPS producing bacteria (Upadhyay et al. 2011). It has reported that the composition of the medium plays an important role in the production of EPS (Vargas-Garcia et al. 2003; Staudt et al. 2012).

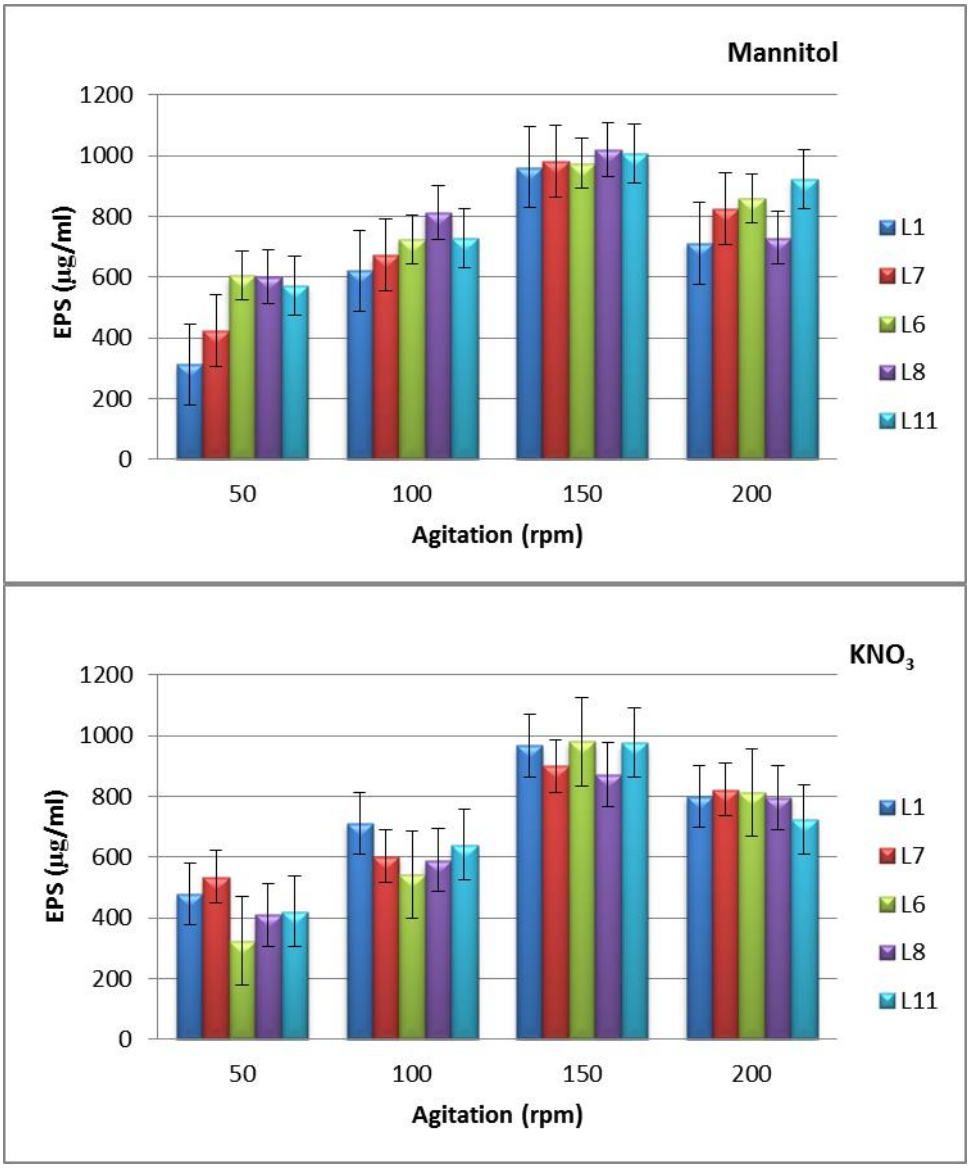

Figure 2. Effect of agitation on EPS production of *Rhizobium* isolates. Error bars indicate Standard deviation of three paralel (p≤0.05)

Breedveld et al. (1993) reported that EPS production can be influenced by carbon and nitrogen sources, pH, incubation time, temperature, agitation. Zevenhuizen (1980), using a mannitol rich culture medium has directed the polysaccharide synthesis towards EPS by applying forced aeration. In regard to aeration, changes in agitation led to a different response for both growth and EPS production (Küçük and Kıvanç 2009). Thus, biomass was generally higher as aeration increased. Oxygen supply is essential for growth of *Rhizobium* bacteria in medium, mainly due to respiratory protection of nitrogenase. As shown in Fig. 2, EPS production by the isolates increased in 150 rpm (Fig. 2). Similar observations were reported by other researchers (Staudt et al. 2012). Limitation of available oxygen is know to influence growth and polysaccharide accumulation by individual organisms in very specific manner. EPS production by *R. meliloti* (Dudman 1964) was reported to be increased significantly in low O_2 supply. EPS produced by *Rhizobium*, bacteria play important roles in protection against the host defense (D'Haeze et al. 2004). If EPS do indeed function as recognition factor, then such substituent changes could affect the host legume specificity. It has been reported that the symbiotic interaction between *Rhizobium* bacteria and legumes have revealed that EPS synthesis by the *Rhizobium* sp. is essential for normal infection tread formation and therefore for the formation of nitrogen fixing nodules on these host plants (Downie 2010; Oldroyd et al. 2011).

In conclusion, *Rhizobium* isolates from *Lathyrus cassius* were able to produce the EPS in the presence of various carbon and nitrogen sources. Although several reports on the importance of EPS in symbiotic plant bacterium interactions have been published (Janczareck et al. 2015), a little information has been gathered about wild *Lathyrus* rhizobia. It is also the first evidence of Turkish wild *Lathyrus cassius* root nodule isolates obtained in this study. In addition, EPS production by these *Rhizobium* isolates could be helping to promote legume-*Rhizobium* infection and enhance nodulation. EPS production helps in

the infection of the host and in subsequent nodulation. Further research on the relationship between isolates and *Lathyrus cassius* and the compositions of EPS produced by *Rhizobium* sp. isolates will be needed to clarify these findings.

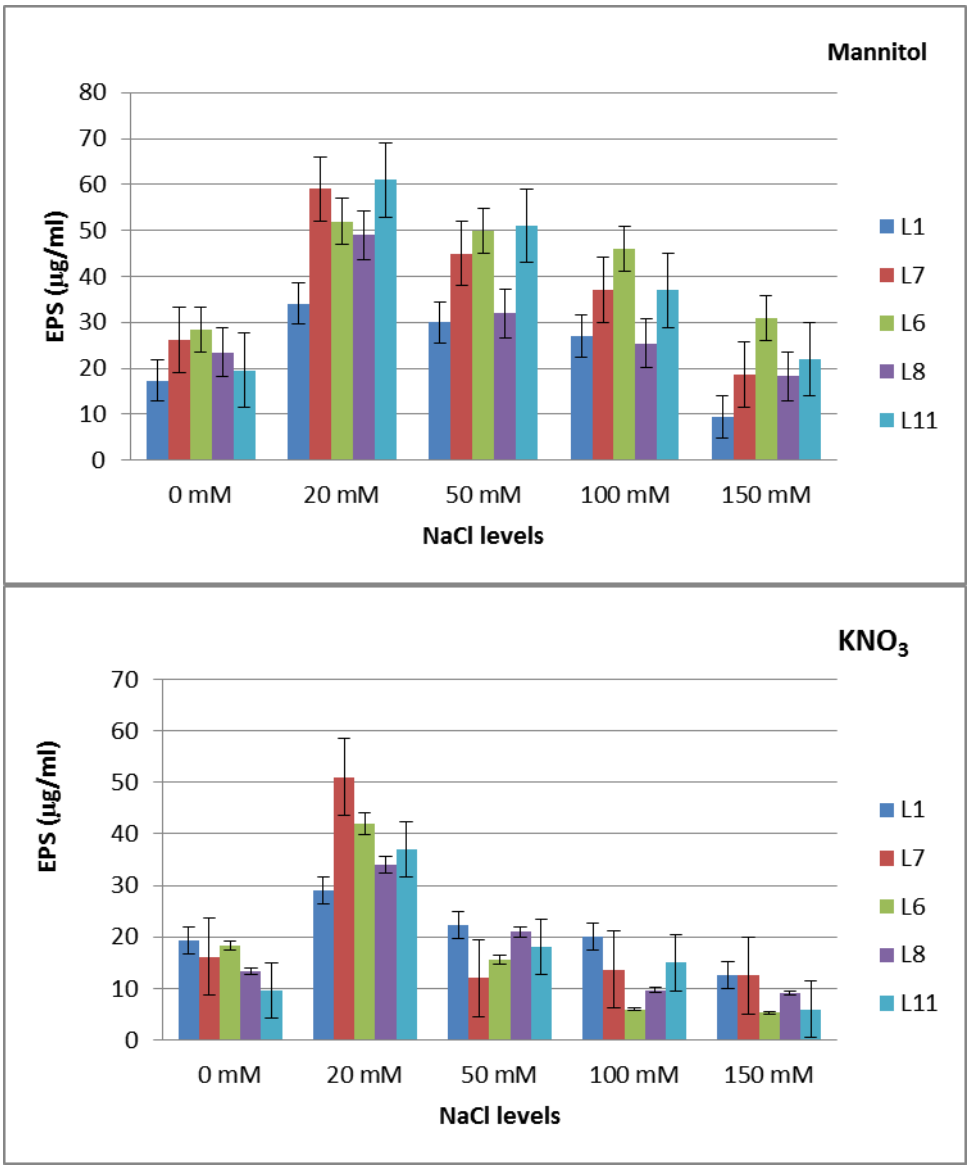

Figure 3. Effect of NaCl on EPS production of *Rhizobium* sp. isolates. Error bars indicate Standard deviation of three paralel (p≤0.05)

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