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Extracellular polysaccharide production by *Rhizobium ciceri* from Turkey

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Abstract - The ability of the *Rhizobium ciceri*, to produce extracellular polysaccharides (EPS) was investigated. Tested carbon and nitrogen sources influced EPS synthesis when *R. ciceri* Rc5 was grown in a chemically defined medium. Mannitol was the most efficient carbon source among the six sources tested (sucrose, glucose, arabinose, fructose, xylose and rhamnose) and sodium nitrate was the most efficient nitrogen source among the two tested (ammonium sulphate and glycine). High amounts of EPS (1182.0 and 1015 µg ml^{-1}, respectively) were produced by the Rc5 strain in mannitol and sodium nitrate respectively, which was accompanied by a great increase in the production compared to the control.

Key words: *Rhizobium ciceri*; strain; exopolysaccharide; carbon and nitrogen source.

Bacteria of the genus *Rhizobium* form a symbiotic nitrogen fixing association with plants in the family leguminosae (Becker and Pühler, 1998). This is a selective interaction, since of plant genera which they can infect and in which they can establish a nitrogen fixing symbiosis (Putnoky *et al*., 1990; Hirsh, 1999). Rhizobia characteristically synthesize copious amounts of extracellular polysaccharides (Kondorosi, 1998; Datta and Basu, 1999). The notion that *Rhizobium* exopolysaccharides (EPS) participate in the inital stages of recognition leading to host legume nodulation through interaction with lectins in root hairs (Leigh and Coplin, 1992; Hirsh, 1999; Ghosh *et al*., 2005) is currenlty a subject of active investigation in many researches. For the cells, 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 (Cunningham and Munns, 1984; Hirsh, 1999). Rhizobial EPS are though to play a role in determinating the host plant specificity of nodulation (Zevenhuizen, 1986; Battisti *et al*., 1992).

EPS characteristics and amounts can be influenced by several factors such as composition of the carbon and nitrogen sources as well as incubation conditions (Duta *et al.,* 2004, 2006). In this work we have studied extracellular polysaccharide production by the *Rhizobium ciceri* Turkish strains and the effect of some sources on production of this substance.

Carbon and nitrogen source assays were carried out in 400 ml of chemically defined medium (0.05% yeast extract, 1% mannitol and 0.01% $CaCI_2{\cdot}2H_2O$, pH 7) (Dudman, 1964). Bacteria were grown (10^8 CFU ml^{-1}) in chemically defined medium containing 10 mM of either sucrose, glucose, arabinose, mannitol, fructose, xylose, rhamnose and containing 0.1% of either glycine, ammonium sulphate, sodium nitrate. Carbon and nitrogen sources was separately sterilized and added to the medium later. The effect of different carbon sources was also studied into the chemically defined medium omitting mannitol. The effect of different nitrogen sources was also studied into the chemically defined medium omitting yeast extract. Production of EPS was make according to Cunningham and Munns (1984) and Becker and Pühler (1998).

A Haeker VT02 model viscometer was used to determine viscosity of strains in different source in growth medium. The pH of the samples was determined using a pH meter. Modifications of agitation (50, 100, 150 and 200 rpm) and pH (5, 6, 7, 8 and 9 pH) were also made (Fig. 1) to study the influence of culture conditions on polysaccharide production. Analysis of variance was obtained following the methods of Düzgüneş *et al.* (1987).

Preliminary experiments were performed in order to determine in the incubation time for optimum recovery of EPSs in *Rhizobium ciceri* strains. Samples were removed at intervals and quantitative extraction of EPS was carried out. According to these results (data not shown), maximum EPS recovery was obtained in 8 day old cultures, so this incubation period was selected for EPS production in further experiments. The formation of polysaccharide was observed in 18 Turkish *Rhizobium ciceri* strains (Table 1). The results show that the viscosity of the polysaccharide varied from one strain the next. In *Rhizobium* Rc5 both the yield and the viscosity were the highest. For this reason, *Rhizobium* Rc5 was selected as the most favorable strain to carry out all subsequent experiments of this investigation (Table 1).

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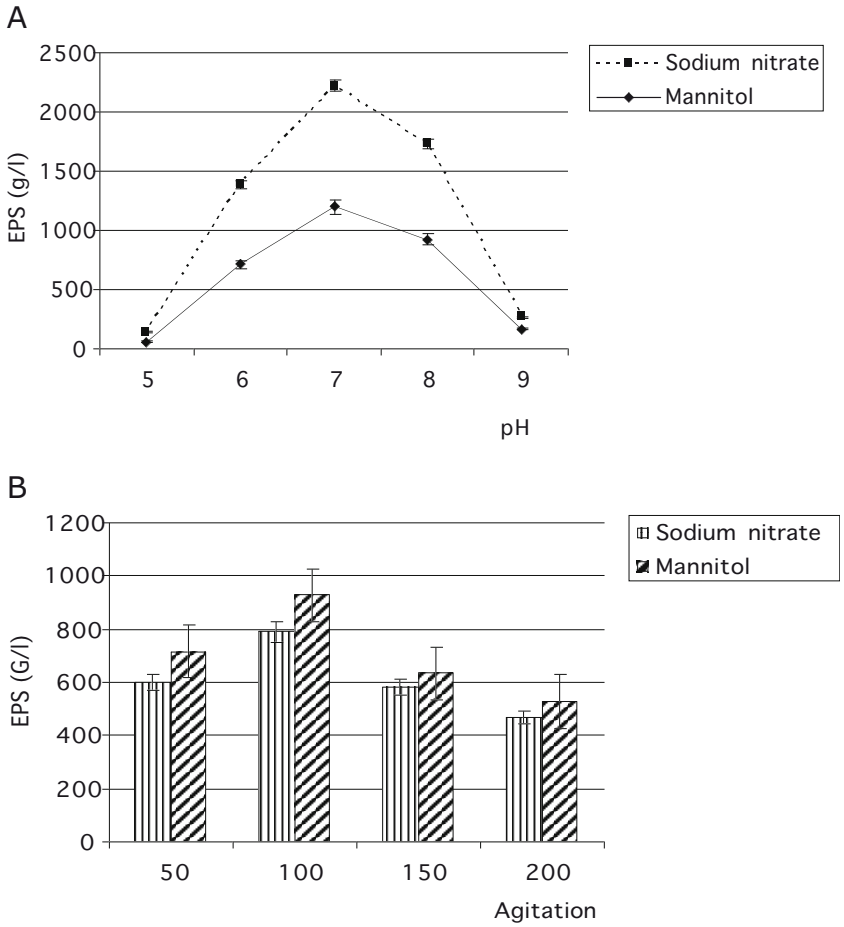

FIG. 1 - Effect of pH (A) and agitation (B) on biomass and EPS production of *Rhizobium ciceri* strain Rc5. Error bars indicate standard deviation of three paralel (p: 0.01).

TABLE 1 - Exopolysaccharide production by various strains *Rhizobium ciceri*

Rhizobium ciceri strain*	Final pH	Viscosity of broth (mPas)	Polysaccharide (g 100 ml^{-1})
Rc1	4.2	1.97	0.82
Rc2	4.0	2.18	1.16
Rc3	3.8	2.09	1.10
Rc 4	5.4	1.60	2.02
Rc 5	5.4	3.25	2.54
Rc 6	5.2	-	-
Rc 7	4.8	-	-
Rc 8	4.4	1.83	0.87
Rc 9	4.0	2.00	1.20
Rc 10	4.9	1.91	1.22
Rc 11	4.3	2.40	1.72
Rc 12	3.2	1.86	0.57
Rc 13	3.0	-	-
Rc 14	4.4	-	-
Rc 15	4.6	2.25	1.10
Rc 16	5.2	2.68	1.17
Rc 17	5.8	2.92	1.10
Rc 18	5.2	2.12	1.08

* *Rhizobium ciceri* strains were obtained from Anadolu University, Microbiology culture collection.

Results for growth and EPS production are shown in Table 2. The strains which initially produced 378.0 µg ml^{-1} EPS in medium whithout additional carbon sources (control). The carbon source affected both dry cell weight and EPS production. Probably, the differences could be explained on the basis of the different nature of the carbon sources employed. Mannitol gave the best result and followed by fructose and glucose. Xylose and rhamnose gave poor yields of polysaccharide (Table 2). The results demonstrate that strain Rc5 is capable of utilizing various sugars for exopolysaccharide production. Breedveld *et al*., (1993) and Bassio *et al.* (1996) reported about the utilization of mannitol by *Rhizobium leguminosarum* for EPS production. Battisti *et al*. (1992) and Datta and Basu (1999) observed the presence of glucose, mannitol and fructose in EPS, which were secreted by the *Rhizobium* strains. In order to improve EPS production by strain Rc5, the influence of nitrogen source was studied (Table 2). As described for the carbon sources assays, the nitrogen source affects both growth and EPS production. Sodium nitrate, glycine and ammonium sulphate showed a maximum production of 1015, 825 and 628.5 µgml^{-1}, respectively. Among these, mannitol and sodium nitrate had greater influence on growth and EPS production. The Rc5, which produced 1182.0 µg ml^{-1} EPS in a medium with mannitol and 1015.0 µg ml^{-1} EPS in a medium with sodium nitrate (increase compared to the control 212.6 and 168.5%, respectively). The results of variance analysis showed that the nitrogen sources and carbon sources were significant.

Medium containing mannitol or sodium nitrate was observed in effect agitation and pH on biomass and EPS production (Fig. 1). To ascertain the effect of pH on cell growth and EPS production, we cultivated the cells in the mannitol or sodium nitrate containing medium having different pH in a flask culture (Fig. 1). In the acidic culture pH, both cell concentration and EPS production was lowered on culture. Similar results have been reported for *Azotobacter vinelandii* grown on sucrose (Vermani *et al*., 1995) and for other EPS producting bacteria (Duta *et al*., 2004, 2006). Although some studies have indicated that environmental conditions do not affect EPS biosynthesis (Jarman *et al*., 1978), others had opposite results (Nandal *et al*., 2005; Duta *et al.,* 2006). pH and agitation have been described as the most influential factors on EPS yields (Vermani *et al.,* 1995). Extreme values of pH resulted in lower EPS yields, especially in the acid range (Fig. 1). Similar results have been obtained in other studies (Duta *et al.,* 2004), and confirmed the necessity to control pH to get optimal substrate uptake and optimal EPS synthesis (Bassio *et al.,* 1996). The process for exopolysaccharides production is carried out under agitation. According to Breedveld *et al*. (1993), for a rise in biomass from aerobic microorganisms a vigorous aeration is required what should be reached by forced aeration. Zevenhuizen (1980), using a mannitol rich culture medium has directed the polysaccharide synthesis towards exopolysaccharides by applying forced aeration. In regard to aeration, changes in agitation changes in agitation led to a different response for both growth and EPS production. Thus, biomass was generally higher as aeration increased. Oxygen supply is essential for growth of *Rhizobium* in medium, mainly due to the respiratory protection of nitrogenase. As shown in Fig. 1, growth was higher as agitation was increased and EPS production was optimal at lower agitation. These results agree with those described by others (Datta and Basu, 1996; Duta *et al*., 2006).

The relationship between exopolysaccharide production by *Rhizobium* strains and the ability to nodulate host plants has been the subject of many studies, most of which were done affected in the synthesis of EPS (Putnoky *et al.,* 1990). If exopolysaccharide do indeed function as recognition factors, then such substituent changes could affect the host legume specificity. EPS production helps in the infection of the host and in subsequent nodulation. The increased EPS production by Rc5 in culture could be helpful in industry as some bacteria which secrete polysaccharides are important to the food, pharmaceutical and oil industry. Although several reports on the importance of EPS in pathogenic or symbiotic plant bacterium interactions have been published (Datta and Basu, 1999; Ghosh *et al*., 2005), our study is first evidence of Turkish *Rhizobium ciceri* strains. Further research on the relationship between *Rhizobium ciceri* Rc5 and host plant and the amounts and compositions of the EPS produced by *Rhizobium ciceri* Rc5 strain will be needed to clarify these findings.

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TABLE 2 - Effect of carbon and nitrogen sources on exopolysaccharide production by *Rhizobium ciceri* strain Rc5 at 192 h culture

Source	Dry cell weight (g ml^{-1})	EPS	
		µg ml^{-1}	Increase (%)*
Carbon			
Sucrose	3.2	710.5	87.9
Glucose	4.2	817.0	116.1
Arabinose	3.0	702.0	85.7
Mannitol	5.7	1182.0	212.6
Fructose	4.8	980.0	159.2
Xylose	2.0	510.5	35.0
Rhamnose	2.2	575.0	52.1
Control	1.8	378.0	100
ANOVA			
	df	Mean square	Mean square
Replication	1	0.0095**	4**
Carbon sources	7	4.04**	134639.8**
Error	7	0.03	1.28

Source	Dry cell weight (g ml^{-1})	EPS	
		µg ml^{-1}	Increase (%)
Nitrogen			
Glycine	3.6	825.0	118.2
Ammonium sulphate	2.5	628.5	66.2
Sodium nitrate	5.3	1015.0	168.5
Control	1.8	378.0	100
ANOVA			
	df	Mean square	Mean square
Replication	1	0.0612**	4.5**
Nitrogen sources	3	4.601**	148217.5**
Error	3	0.022	0.17

* Compared to the control.

** Significant at the 0.01 probability levels.

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