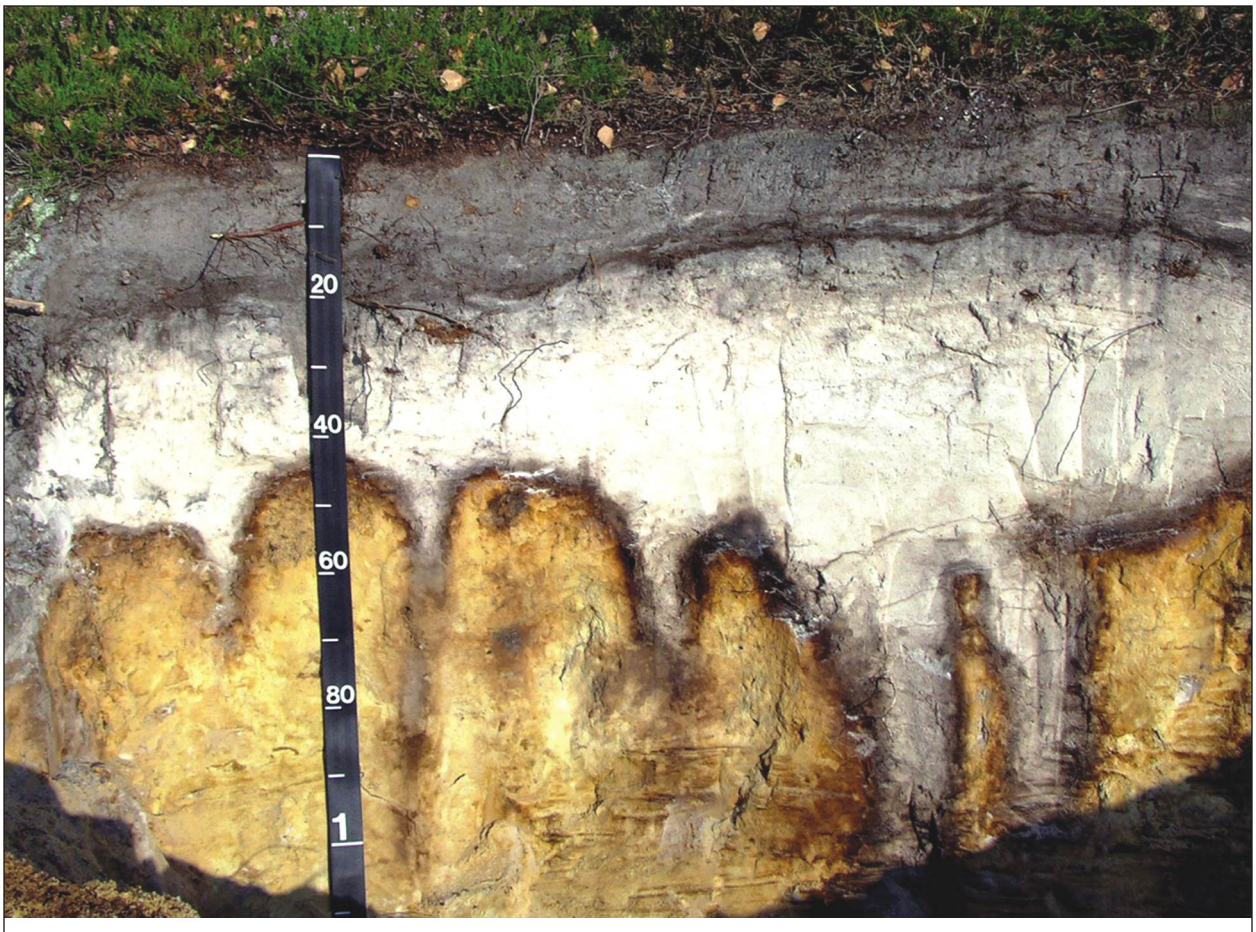

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Production of Extracellular Polysaccharide by *Rhizobium* Strains from Root Nodules of Leguminous Green Manure Crop, *Sesbania sesban* (L.) Merr.

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Abstract: The ability of twenty six *Rhizobium* strains, isolated from root nodules of leguminous crop, *Sesbania sesban* (L.) Merr. were tested for their production of extracellular polysaccharides (EPS) in Yeast Extract Mannitol (YEM) medium. Among the twenty six strains, maximum amount of EPS was produced by *Rhizobium* SS5 strain (3900 μg mL^{-1}). Both growth and EPS production started simultaneously, but the EPS production was maximum in the stationary phase of growth (48 h). The EPS production was maximum, when the medium was supplemented with galactose (2%), sodium nitrate (0.1%) and Ca-pantothenate (1 μg mL^{-1}), which was accompanied by a great increase in the production compared to the control. The EPS contained galactose, glucose, xylose, rhamnose and raffinose, which were identified by paper as well as gas liquid chromatography.

Key words: Extracellular polysaccharides, *Rhizobium* species, *Sesbania sesban*, legume-*Rhizobium* association, gas-liquid chromatography

INTRODUCTION

Many physiological aspects of *Rhizobium*-legume symbiosis are still poorly understood, although rhizobial root nodules of leguminous plants have created great interest among scientists for a long period of time. The production of rhizobial extracellular polysaccharides (EPS) is one of these aspects. Rhizobial EPS was shown to be involved in the *Rhizobium*-legume symbiosis. Olivares *et al*. (1984) reported about the enhancement of nodulation by EPS. *Rhizobium* species were described which were able to produced EPS also in culture (De and Basu, 1996; Ghosh *et al*., 2005). Skorupska *et al*. (2006) also reported that extracellular polysaccharides may be involved in invasion and nodule development, bacterial release from infection threads, bacteroid development, suppression of plant defense response and protection against plant antimicrobial compounds.

Sesbania sesban (L.) Merr. is a widely cultivated green manure crop in Andhra Pradesh, India. Very little information about the EPS production by the symbiont of *S. sesban*. The objective of this study was to screen the maximum EPS producing strain from *S. sesban* and also to increase the production of EPS through optimization of cultural conditions of the strain, which produced maximum amount of EPS.

MATERIALS AND METHODS

Microorganism, Medium and Growth Conditions

Twenty six *Rhizobium* strains were isolated from fresh healthy root nodules of *Sesbania sesban* collected from different regions of Andhra Pradesh, India. The study was conducted in December, 2006 in the Department of Microbiology, Acharya Nagarjuna University. The basal medium for the bacterial

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growth and EPS production were the yeast extract mineral medium (Skerman, 1959) with 1% mannitol. The strains were incubated in 25 mL of the medium in 100 mL conical flasks in three replicates at 30±2°C for 48 h (optimum time for maximum EPS production). The growth was measured spectrophotometrically at 540 nm.

Production of EPS on Different Sources

Different carbon sources were added separately to the basal medium replacing mannitol. Individual effect of different chemicals with most suitable carbon source on EPS production was tested. For maximum EPS production by the strain the medium was enriched with different supplements which individually increase the EPS production to maximum level. All the supplements added to the medium were filter sterilized.

Isolation of EPS

Isolation of EPS was done by following the method described by Dudman (1976) and collected by centrifugation, dissolved in minimum volume of distilled water, reprecipitated with 3 volumes of acetone, centrifuged, dialyzed and lyophilized. For identification of sugar monomers, dry EPS was hydrolyzed in a sealed tube with 0.5 M $BaCO_3$ and concentrated at 45°C under reduced pressure. EPS was chromatographed on Whatman No. 1 paper using butanol: acetic acid: water (4:3:1) as solvent system. Spraying reagent used for identification of sugar components was aniline phthalate (Partridge, 1949). For Gas Liquid Chromatography (GLC), sugar derivatives (paracetylated alcohols) were prepared from dry lyophilized polysaccharides (Ghosh *et al*., 2005) and injected into GLC apparatus. The sugar derivatives were identified by comparisons of their retention times with those of authentic standards.

Estimation of EPS

The dialyzed cell free supernatant was used for EPS estimation by phenol-sulphuric acid method following Dubois *et al*. (1956). Uronic acid estimation in the EPS was performed by Carbazole reaction (Dische, 1947).

Statistical Analysis

The data were statistically analyzed using correlation coefficient between growth and EPS production.

RESULTS AND DISCUSSION

The *Rhizobium* strains isolated from root nodules of *S. sesban* were designated as *Rhizobium* SS1 to SS26. The isolated strains were identified as species of *Rhizobium* following Bergey's Manual of Systematic Bacteriology (Jordan, 1984) and plant infection test (Vincent, 1970). The *Rhizobium* strains were fast growers and reached stationary phase at 48 h. Among the 26 *Rhizobium* strains tested, the *Rhizobium* SS5 produced maximum amount of EPS on yeast extract mannitol medium (Table 1). Maximum EPS production was also observed at 48 h by this strain. As *Rhizobium* SS5 produced more amounts of EPS, further tests were carried out on this strain.

All the fourteen carbon sources (1%) promoted both growth and EPS production, but maximum amount was observed in galactose followed by mannitol (Table 2). But the mannitol was the best carbon source was reported earlier in *Rhizobium* D110 sp. from *Dalbergia lanceolaria* (Ghosh *et al*., 2005). The optimum concentration of galactose required for EPS production was found to be 2.0% (Table 4).

Table 1: Production of Extracellular polysaccharides (EPS) by *Rhizobium* strains from *Sesbania sesban*

Strains	Growth* (OD at 540 nm)	EPS production* (μg mL^{-1})	Specific productivity (EPS production/growth)
Rhizobium SS1	1.40	2800	2000.0
Rhizobium SS2	1.30	2500	1923.0
Rhizobium SS3	1.20	2550	2125.0
Rhizobium SS4	1.10	1800	1636.3
Rhizobium SS5	1.25	3900	3120.0
Rhizobium SS6	1.50	2800	1866.6
Rhizobium SS7	1.50	2800	1866.6
Rhizobium SS8	1.25	2000	1600.0
Rhizobium SS9	1.20	2000	1666.6
Rhizobium SS10	1.30	2500	1923.0
Rhizobium SS11	1.00	2000	2000.0
Rhizobium SS12	1.35	2900	2148.1
Rhizobium SS13	1.30	3000	2307.6
Rhizobium SS14	1.20	2800	2333.3
Rhizobium SS15	1.30	1900	1461.5
Rhizobium SS16	1.40	2000	1428.5
Rhizobium SS17	1.35	2500	1851.8
Rhizobium SS18	1.30	2200	1692.3
Rhizobium SS19	1.20	2500	2083.3
Rhizobium SS20	1.20	2900	2416.6
Rhizobium SS21	1.20	2900	2416.6
Rhizobium SS22	1.40	2000	1428.5
Rhizobium SS23	1.00	800	800.0
Rhizobium SS24	1.00	800	800.0
Rhizobium SS25	0.80	520	650.0
Rhizobium SS26	0.90	200	222.2

*: Correlation coefficient between growth and extracellular polysaccharide production (r = 0.71)

Table 2: Effect of different carbon sources on growth and extracellular polysaccharide production by *Rhizobium* SS5

Carbon source	Growth* (OD at 540 nm)	EPS production* (μg mL^{-1})	Specific productivity (EPS production/growth)
Control*	0.90	600	666.6
Mannitol	1.25	3900	3120.0
Glucose	1.20	3700	3083.3
Galactose	1.75	6400	3657.2
Raffinose	0.90	2800	3111.1
Xylose	1.25	3500	2800.0
Inositol	0.90	2500	2777.7
Fructose	0.90	2750	3055.5
Lactose	0.80	1950	2437.5
Maltose	0.80	1800	2250.0
Sucrose	1.20	2400	2000.0
Ribose	1.40	2000	1428.5
Trehalose	1.00	800	800.0
Arabinose	1.30	1900	1461.5
Mannose	1.00	2000	2000.0

*: Control was devoid of carbon source; Correlation coefficient between growth and extracellular polysaccharide production (r = 0.67)

Among the nitrogen sources tested, maximum EPS production was observed in sodium nitrate followed by potassium nitrate (Table 3). The optimum concentration was found to be 0.1% (Table 4). But, potassium nitrate at 0.1% concentration increase EPS production in *Rhizobium* D110 strain from *Dalbergia lanceolaria* (Ghosh *et al.*, 2005).

Among the different vitamin sources tested, Ca-pantothenate was most effective source for maximum EPS production at 1 μg mL^{-1} (Table 3). But, D-Biotin at 1 μg mL^{-1} increased both growth and EPS production were reported in *Azorhizobium caulinodans* from *Aeschynomene aspera* (Ghosh and Basu, 2001).

Table 3: Effect of different nitrogen sources and vitamins on growth and extracellular polysaccharide production by *Rhizobium* SS5

Sources	Growth* (OD at 540 nm)	EPS production* (μg mL^{-1})	Specific productivity (EPS production/growth)
Nitrogen			
Control*	1.85	3020	1632.4
Ammonium sulphate	1.60	2000	1250.0
Potassium nitrate	1.90	3900	2052.6
Sodium nitrate	2.92	7500	2568.4
Ammonium chloride	1.65	2500	1515.2
Glycine	1.50	1500	1000.0
Glutamic acid	1.55	1900	1225.8
L-asparagine	1.42	1200	845.0
Casamino acid	1.45	1250	862.0
Vitamins			
Control*	1.85	3500	1891.9
Riboflavin	1.85	3500	1891.9
Thiamine HCl	1.85	3500	1891.9
Nicotinic acid	1.90	3900	2052.6
Pyridoxal phosphate	2.50	4200	1680.0
Ca-pantothenate	2.55	8300	3254.9
Ascorbic acid	2.00	4300	2150.0
Biotin	2.05	4200	2048.7

*: Control was devoid of any type of additional nitrogen and vitamin sources; Correlation coefficient between growth and extracellular polysaccharide production in nitrogen sources (r = 0.99), in vitamins (r = 0.74)

Table 4: Effect of different concentrations of galactose and sodium nitrate on growth and extracellular polysaccharide production

Concentrations (%)	Growth (OD at 540 nm)	EPS production (μg mL^{-1})
Galactose concentration		
1.0	1.85	3020
2.0	1.90	4250
3.0	1.90	3900
Sodium nitrate concentration		
0.05	1.85	3520
0.10	1.90	4050
0.15	1.90	3000
0.20	1.50	2500

Table 5: Increase in growth and extracellular polysaccharide production by *Rhizobium* SS5 using most effective supplements

	Growth		EPS	
Supplements	(OD at 540 nm)	% increased compared to control	μg mL^{-1}	% increased compared to control
Control*	0.90	--	600	--
Galactose	1.75	94.4	6400	966.6
Galactose+Ca-pantothenate+ Sodium nitrate	3.00	233.3	7200	1100.0

*: In the control, bacteria were grown on yeast extract mannitol medium. In other cases the medium was supplemented with galactose (2%), Ca-pantothenate (1 μg mL^{-1}) and sodium nitrate (0.1%)

To test the maximum EPS production by *Rhizobium* SS5 strain in culture, the supplements which individually increased the production to the greater extent was added to the medium. The strain which initially produced 3900 μg mL^{-1} EPS in basal YEM medium was induced to yield more amounts of EPS through optimization of cultural conditions (Table 5). The EPS produced by this strain contained galactose, glucose, xylose, rhamnose and raffinose, which were identified by paper and gas liquid chromatography. The sugar isomers contained 30.2% galactose, 20.9% glucose, 20.4% xylose, 16.3% raffinose and 12.2% rhamnose, taking total of the five sugars as 100% (Table 6).

Table 6: Relative (%) of sugar monomers in the extracellular polysaccharides of *Rhizobium* SS5 as identified by GLC

Sugar monomers	Relative (%)
Galactose	30.2
Glucose	20.9
Xylose	20.4
Rhamnose	16.3
Raffinose	12.2

Hollingsworth *et al*. (1985) also observed the presence of galactose, glucose and mannose in EPS, which were secreted by *Rhizobium* strain of M1-50A, M6-78 and IRC 253 of cowpea rhizobia. EPS of some members of *Rhizobiaceae* contains mannitol and fructose (Breedveld *et al*., 1993). These have indicated that there are variations in the sugar monomers from different *Rhizobium* spp.

The EPS secreted by *Rhizobium* SS5 was acidic, indicating the presence of uronic acid. The amount of uronic acid was found to be 352.9 μg mL^{-1} of EPS. Amemura *et al*. (1983) have reported that most extracellular acidic polysaccharides of *Rhizobium trifolii* contained D-glucuronic acid.

Correlation between the growth and EPS production in YEM medium is positive (r = 0.71). The effect of carbon, nitrogen and vitamin sources also showed positive correlation that of nitrogen sources is highly positive (r = 0.99).

CONCLUSIONS

All the supplements which increased the EPS production in culture could be available for the *Rhizobium* SS5 in the soil from the plant as root leachate. This might stimulate the *Rhizobium* to produce more polysaccharides helping to promote the infection and enhance nodulation of legumes (Ghosh *et al*., 2005). Moreover, the increased EPS production by the strain SS5 could be useful for the industry. Glycan, dextran and xanthan of bacterial origin are of commercial importance.

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