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Effect of carbon and nitrogen sources on exopolysaccharide production by rhizobial isolates from root nodules of *Vigna trilobata*

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Twenty five (25) rhizobial strains were isolated from root nodules of *Vigna trilobata* cultivars grew in soils collected from all districts of Andhra Pradesh, India. Five out of 25 rhizobial strains which produced copious amount of Exopolysaccharides (EPS) on Yeast Extract Mannitol Agar (YMA) medium with congo red were identified by sequencing of their 16S rDNAs. The amount of EPS produced by these five strains increased during the first 72 h of incubation but it declined afterwards. The amount of EPS produced correlated positively with increase in mannitol concentration from 1 to 3% (m/v). However, there was a decrease in EPS production when mannitol concentration was equal or higher than 4%. All the five strains studied preferred mannitol and sodium nitrate as the best carbon and nitrogen sources for EPS production. *Sinorhizobium kostiense* MRR104 produced maximum levels of EPS 892 mg/100 ml when mannitol was used as carbon source, while *S. xinjiangense* MRR110 produced maximum levels of EPS 377 mg/100 ml when sodium nitrate was used as nitrogen source. Variation among the rhizobial strains in utilization of carbon and nitrogen sources for EPS production was clearly evident from this study. All the strains analysed in the present study can be exploited for production of commercial biopolymer (gum), due to their non pathogenic nature and their ability to produce copious amounts of EPS when compared to strains studied in earlier reports.

Key words: Rhizobial strains, *V. trilobata,* Exopolysaccharides (EPS), carbon and nitrogen sources.

INTRODUCTION

Rhizobium spp. are known to synthesize a variety of cell surface polysaccharides, including exopolysaccharides (EPS), lipopolysaccharides (LPS), and cyclic glucans (CG). In addition, *Sinorhizobium* spp. produces K-antigen polysaccharides (KPS). All these four polysaccharides are important for bacterial performance in both free life and symbiosis (Margaret et al., 2011). Rhizobial exopolysaccharides (EPS) play an important role in plant root invasion during nodule formation and promotes growth of the plants by chelating various metal ions. EPS helps in creation of near anaerobic conditions in the microenvironment surrounding the rhizobial cell surface,

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before infecting the root hair, to protect the nitrogenase enzyme (Gupta et al., 1982) and enhances nodulation (Olivares et al., 1984). EPS protects the producing organism against desiccation (Sayyed et al., 2011) toxic compounds, and osmotic stress, helps in the formation of biofilms (Kucuk and Kivanc, 2009) and serves as energy source to be catabolised during nutrient deficiency. Apart from its role in symbiosis and plant growth, the EPS synthesized in culture have importance in many industries including food, oil and pharmacy. Microbial EPS have been commercialized as possible future industrial commodities for food as thickening agents and in agriculture for the encapsulation of somatic embryos, which offer a greater feasibility for precise delivery of plant growth regulators, fungicides and pesticides (Mathur and Mathur, 2001). EPS has been proved to be a potential biopolymer used as emulsifier in the degradation of hydrocarbons, stabilizers, binders, coagulants and separating agents in a variety of industries (Staudt et al., 2011). Bacterial EPS exhibit antioxidant activity (Mahendran et al., 2013). Recently rhizobial EPS were patented as best bioproduct for skin treatment (US Patent 20,130, 302, 261- 2013). EPS produced by bacteria are commercially exploited so far, however, much of the present day research is concentrating on the industrial application of rhizobial EPS. Rhizobial biopolymers are preferred in the food industry by virtue of their non pathogenic nature and copious production (Bomfeti et al., 2011). Rhizobial EPS are species or strain specific hetero polysaccharides consisting of repeating units of sugars (Mandal et al., 2007) the majority of which are hexoses and uronic acids as well as non carbohydrate substituents such as acetate, pyruvate, hydroxyl butyrate and succinate (Aman et al., 1981; Cunningham and Munns, 1984). However, the most common types of EPS produced by Rhizobia are EPS1 and EPS2. EPS1 type are high molecular weight succinoglycans and EPS II are low molecular weight galactoglucans (Oliveira et al., 2012). Several factors influence the production of EPS by rhizobial strains, such as carbon and nitrogen sources as well as incubation period (Duta et al., 2004, 2006).

Vigna trilobata commonly called Pillipesara, was mainly cultivated as short term pasture and green manure crop in India, Pakistan, Indonesia and Sudan. Though nodulation in *Vigna trilobata* was first reported in Japan by Asia and in India by Raju in 1936 (Allen and Allen, 1981) the comprehensive studies on rhizobial symbiont characterization and other related studies are meagre. The present investigation was aimed to study the diversity of rhizobial strains producing exopolysaccharide. Although much work was published on EPS production, characterization and factors effecting EPS production by rhizobial strains from different host legumes, our study is the first of its kind on rhizobial strains isolated from nodules of *V. trilobata.* In this paper, for the first time, rhizobial species nodulating V*. trilobata* have been identified by 16S rDNA sequencing.

MATERIALS AND METHODS

Isolation of rhizobia

Soil samples were collected from agricultural fields under the cultivation of *V. trilobata* from all the 25 districts of Andhra Pradesh. Certified seeds of *V. trilobata* were purchased from the National Seed Corporation (NSC) Guntur. Plants were grown in earthen pots filled with these district soils and were maintained properly in the Botanical garden of Acharya Nagarjuna University. After 90 days of germination, healthy root nodules from gently uprooted plants, surface sterilized with 0.1% mercuric chloride and 70% alcohol and washed thoroughly by sterile distilled water were used for isolation (Vincent, 1970). Rhizobial strains were isolated from root nodules of *V. trilobata* plants, using selective medium Yeast Extract Mannitol Agar (YMA) with congo red and pure cultures were maintained after sub culturing on the same medium. Pure cultures of all the 25 isolates were authenticated as rhizobia by performing the appropriate biochemical tests (Somasegaran and Hoben, 1994) and nodulation ability on homologous hosts by plant infection tests (Vincent, 1970). Out of the 25, the five strains which produced higher amounts of EPS were further identified up to the species level through 16S rDNA sequencing (Macrogen, South Korea) and the sequences were deposited in the gene bank. The strain names with allotted accession numbers, used in this study are *Rhizobium sp.*MRR103-JX576499 (isolated from Guntur district soil); *Sinorhizobium kostiense* MRR104 - KC428653 (isolated from Chittor district soil); *Sinorhizobium xinjiangense*MRR110 -KC415691 (isola-ted from Kadapa district soil); *Rhizobium sp.*MRR 123 - KC503884 (isolated from Nellore district soil); *Ensifer sp.*MRR125 - KC503885 (isolated from Mahaboobnagar district soil).

Exopolysaccharide (EPS) production

For production of EPS, all the five strains were inoculated into Erlenmeyer flasks (250 ml) containing 100 ml of YMB supplemented with 1% Mannitol (m/v). The flasks were incubated at room temperature on an orbital shaker at 200 rpm for 72 h. After incubation, the broth was centrifuged at 3000 x *g* and the culture supernatant was mixed with 2 volumes of chilled acetone. The crude polysaccharide precipitated was collected by centrifugation at 3000 x *g* for 30 min. The EPS was washed with distilled water and acetone alternately, transferred into a filter paper and weighed after overnight drying at 105°C (Damery and Alexander, 1969). The strain that produced maximum amount of EPS, *S. kostiense* MRR104 was used in the studies on optimization of the conditions for maximum EPS production, like the incubation period and the concentration of mannitol (carbon source in the YMA medium).

Effect of mannitol concentration

To optimize the concentration of Mannitol for maximum production of EPS production, the test cultures were inoculated with five different concentrations of Mannitol (1, 2, 3, 4 and 5 %) including the concentration prescribed in the original YMB medium (1%). All the inoculated flasks were incubated for 72 h at room temperature on an orbital shaker at 200 rpm and the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

Effect of incubation period

For the study of EPS production at different incubation periods, the test culture was inoculated into YMB medium (1% mannitol) and incubated at different periods from 12 to 84 h with 12 h intervals. The

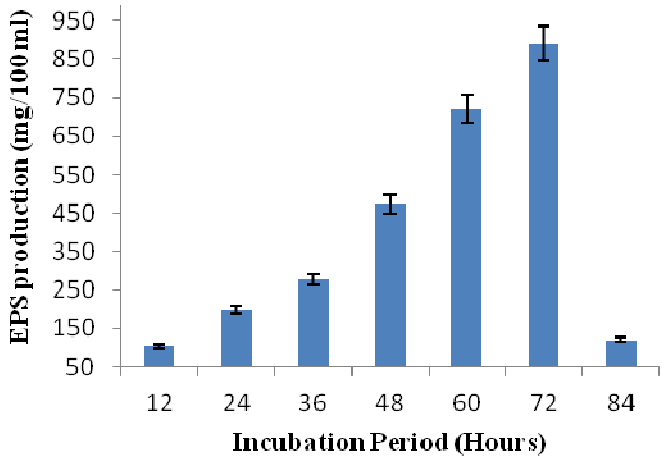

Figure 1. Effect of incubation period on EPS (mg/100 ml) **production by** *Sinorhizobium kostiense* MRR104.

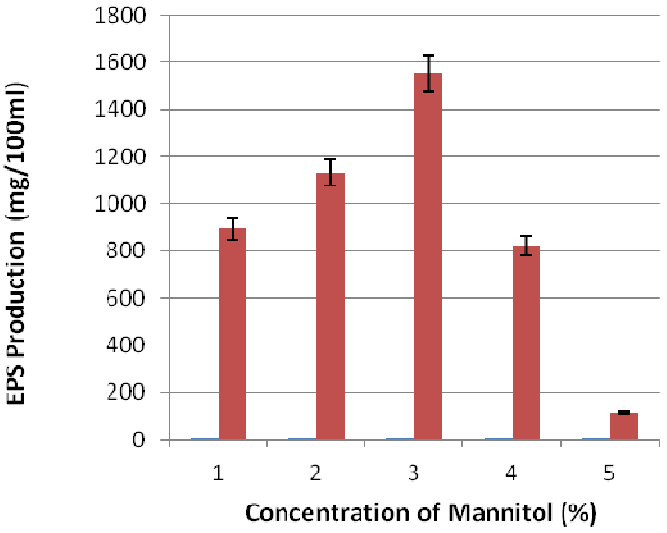

Figure 2. Effect of Mannitol concentration on EPS (mg/100 ml) production by *Sinorhizobium kostiense* MRR104 after 72 hof incubation.

The inoculated flasks were incubated at room temperature on an orbital shaker at 200 rpm and the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

Effect of Carbon and Nitrogen sources on EPS production

To study the effect of carbon source on production of EPS by all the five strains of rhizobacteria, ten carbon sources including six monosaccharides – arabinose, galactose, glucose, fructose, raffanose and xylose; three disaccharides – sucrose, maltose, lactose and one sugar alcohol – mannitol, were used in by replacing, in same concentration (1%), the mannitol in the original YMA medium. Control was maintained without any carbon source. All the five strains viz., MRR 103, MRR 104, MRR 110, MRR 123, MRR 125 which produced maximum EPS were used in this study. Rhizobial cultures were inoculated separately into 100 ml of YMB medium containing different carbon sources and the flasks (250 ml) were incubated on a orbital shaker at 200 rpm for 72 h. After incubation, the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

To study the effect of nitrogen source on the production of EPS, five different nitrogen sources - ammonium sulphate, glycine, sodium nitrate, potassium nitrate, and L-asparagine were selected. All the nitrogen sources were added to the medium by replacing the 0.1% yeast extract of the original YMA medium composition. Rhizobial strains were inoculated separately into the flasks (250ml) containing 100ml of YMB supplemented with different nitrogen sources. All the inoculated flasks were incubated for 72 h on an orbital shaker at 200 rpm. After incubation the amount of EPS produced was estimated by the method described by Damery and Alexander (1969).

Three replicates were used for each treatment. Statistical analyses of the data were performed using SPSS software (version 20). Correlation coefficient and ANOVA were calculated for the data wherever necessary. Duncan's test was used for multiple range analyses to determine the significant difference between groups of data. The results were considered to be significant at $P<0.05$.

RESULTS AND DISCUSSION

In the present study, all the 25 rhizobial strains isolated from *Vigna triolobata* produced EPS by utilizing the available carbon source in the YMB medium. Five out of 25 strains, producing copious amounts of EPS, were selected for further analyses. Fernandes et al. (2011) reported that six out of 38 isolates from *Cajanus cajan* produced more than 200 mg EPS/L. However, our strains produced much higher amounts of EPS (>600 mg/100ml) than those isolated from *C. cajan.* In *S. kostiense* MRR104, EPS production was minimum at 12 h of incubation and the maximum was recorded at 72 h of incubation (Figure 1) and decreased at 84 h. Hence, a period of 72 h of incubation was considered as optimum for maximum EPS production by the isolates of *V. trilobata*. In our experiments, it was statistically proved that incubation period positively correlated with growth (r=0.59) and EPS production (r= 0.47) during the first 72 h. This is not the case for other rhizobial strains studied previously. Kucuk and Kivanc (2009) reported a maximum EPS production after an incubation period of 8 days for *Rhizobium ciceri*, while 6 days was reported by Sayyed et al. (2011) for *Rhizobium* sp. Mannitol as good carbon source supported the rhizobial strains in growth and production of EPS. In the present study, the EPS production increased gradually when mannitol concentration was increased up to 3% and showed a decline from 4% onwards (Figure 2). *S. kostiense* MRR104 produced maximum amount of EPS 1550 mg/100 ml at 3% of mannitol and a minimum (112 mg/100 ml) at 5% mannitol. This decrease in EPS may be due to the mobilization of EPS by the organism itself probably under the influence of EPS hydrolase (Basu and Ghosh, 1999). The influence of mannitol concentration and period of incubation varies among different rhizobial strains. Thus, Mukherjee

Table 1. EPS (mg/100ml) produced by rhizobial strains in YMB supplemented with different carbon sources.

Carbon sources (1.0%)	*Rhizobium* sp. MRR 103	*Sinorhizobium kostiense* MRR 104	*Sinorhizobium xinjiangense* MRR 110	*Rhizobium* sp. MRR 123	*Ensifer* sp. MRR 125
Control	5	14	21	10	21
Mannitol	723	892	692	816	719
Arabinose	364	243	266	305	166
Galactose	307	249	266	171	196
Sucrose	228	285	184	360	122
Maltose	286	397	208	377	378
Lactose	239	319	218	266	226
Glucose	454	650	262	720	412
Fructose	302	311	281	228	293
Raffinose	388	237	270	242	116
Xylose	114	137	149	106	185

et al., (2011) reported that EPS production by *Rhizobium* sp. isolated from *Crotalaria saltiana* was maximum at 2% (m/v) of mannitol, whereas Datta and Basu (1999a) described that EPS production by *Rhizobium* sp. isolated from *C. cajan* reached its maximum at 4% (m/v) of mannitol with an incubation period of only 65 h.

All the five rhizobial strains utilized the 10 different carbon sources and produced significantly high amount of EPS than when a control medium without carbon source was employed (Table 1). Though all the carbon sources supported EPS production, maximum production was observed when sugar alcohol - mannitol was used as carbon source indicating that mannitol supported maximum EPS production by all the strains of *V. trilobata.* This is in agreement with previous reports by Ghosh et al. (2005a) in *Rhizobium* sp. from *Dalbergia lanceolaria,* Kucuk and Kivanc (2009) in *R. ciceri*Rc5 and Mandal et al. (2007) in *Rhizobium* sp. from *Vigna mungo*. However, it has been also described that other rhizobial species show maximum EPS production using different carbon sources. Glucose was preferred as carbon source for maximum EPS production by rhizobial sp. from *Crotalaria saltiana* (Mukherjee et al., 2011) and rhizobial sp. from *Melilotus alba* (Datta and Basu,1999b) while xylose was preferred by isolates from *C. cajan* (Fernandes et al., 2011) and galactose by *Rhizobium* sp. SS5 from *Sesbania sesban* (Sridevi and Mallaiah, 2007). Ghosh et al. (2011) reported that sucrose (1.5%) induced the maxi-mum EPS production though maximum growth was observed with mannitol by *Rhizobium* sp. from *Phaseolus mungo.* Among the monosaccharides tested, after manni-tol, glucose was preferred by most of the strains in the present study while Nirmala et al. (2011) reported that sucrose was preferred next to mannitol by *Rhizobium* sp. from *V. mungo*. Among the disaccharides used, maltose was preferred next to glucose by MRR 104, MRR 123 and MRR 125 strains. From the present study, it is evident that rhizobial strains isolated from *V. trilobata* preferred sugar alcohol, mannitol for maximum EPS production and gave second preference to monosac-charide and less to the disaccharide carbon sources. Among the five strains studied, *S. kostiense* MRR104 produced maximum EPS of 892 mg/100 ml followed by *Rhizobium* sp. MRR 123 with 816 mg/100 ml.

Variation in EPS production by *Aschenomenon aspera* isolates when different carbon sources used was previously reported by Ghosh et al. (2005b). Similarly, significant variations in the amount of EPS produced among the different carbon sources, among the strains and also within the strains were recorded in the present study with the strains of *V. trilobata*.

Statistically there were significant differences between the carbon sources used and rhizobial strains in the production of EPS (p = <0.05). The Duncan test reveals that the highest EPS production occurred for *S. kostiense* MRR104 when mannitol was used as carbon source.

Effect of different nitrogen sources was studied by replacing 0.1% Yeast extract of the original YMA medium with five different nitrogen sources. All the strains studied efficiently utilized those different nitrogen sources and produced high amount of EPS over the control (Table 2). This clearly shows the significant role played by the nitrogen source on EPS production. Sodium nitrate was preferred for maximum production of EPS followed by potassium nitrate by all the strains studied. Similar results were reported previously by Kucuk and Kivanc (2009) in *R. ciceri,* Sridevi and Mallaiah, (2007) in *Rhizobium* sp. SS5 from *Sesbania sesban*. In contrast, potassium nitrate was preferred by *Rhizobium* sp. from *V. mungo* (Nirmala et al., 2011), rhizobial sp. from *Melilotus alba* (Datta and Basu, 1999b) and *Rhizobium* sp. from *Dalbergia lanceolaria* (Ghosh et al., 2005a).

The maximum EPS production of 377 mg/100 ml was recorded with strain *S. xinjiangense* MRR110 followed by 325 mg/100 ml by *Ensifer* sp. MRR125. Significant differences (P = <0.05) between the nitrogen sources on EPS production were recorded in the present study. The Duncan's test reveals that strain *S. xinjiangense* MRR110

Table 2. EPS (mg/100 ml) produced by rhizobial strains in YMB supplemented with different nitrogen sources.

Nitrogen sources (0.1%)	*Rhizobium sp.* MRR103	*Sinorhizobium kostiense* MRR 104	*Sinorhizobium xinjiangense* MRR110	*Rhizobium sp* MRR123	*Ensifer sp.* MRR125
Control	0.005	0.041	0.008	0.006	0.022
Ammonium Sulphate	133	207	165	141	130
Glycine	72	120	92	118	80
Sodium Nitrate	197	284	377	272	325
Potassium Nitrate	167	214	202	203	102
L-Asparagine	80	121	176	111	111

produced maximun EPS when sodium nitrate was used as nitrogen source. Glycine and L- asparagine were less preferred by the strains in the present study. In contrast, *Rhizobium* sp. from *Phaseolus mungo* preferred glycine (Ghosh et al., 2011) and *Rhizobium* sp. from *Vigna mungo* preferred L-asparagine (Mandal et al., 2007) for maximum production of EPS. In the present study, *S. xinjiangense* MRR110 proved to be a more efficient EPS producer than those described in earlier reports of *Sinorhizobium* TR1 from *Trigonella foenum-graecum* (Tank and Saraf, 2003) which produced only 20 μgml^{-1}. Thus, the fact that rhizobial strains exhibit high variations in utilization of carbon and nitrogen sources for the production of EPS was proved among the strains isolated from *V. trilobata* cultivars by the present study. This variation can be attributed to the strain adaptability to the different environmental conditions that prevailed in the soil at geographically different areas from which they were isolated. From the present investigation, it is evident that the rhizobial strains from *V. trilobata* with high EPS production definitely could be added to the list of very few rhizobial strains –*Mesorhizobium pluriforuium* BR3804 with 1.44 g/l and *Rhizobium tropici* CIAT899 with 3.0 g/l EPS production, which were identified as most suitable for commercial production of gum (Bomfeti et al., 2011).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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