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ORIGINAL RESEARCH ARTICLE

Production of Extracellular Polysaccharides by a *Rhizobium* Species from Root Nodules of *Vigna mungo* (Hepper)

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ABSTRACT

The ability of ten *Rhizobium* strains, isolated from root nodules of leguminous crop, *Vigna mungo* (L.) Hepper were tested for their production of Extracellular Polysaccharides (EPS) in Yeast Extract Mineral (YEM) medium. Among the ten strains, maximum amount of EPS was produced by *Rhizobium* Vm6 strain (1680 µg/mL). The maximum amount of EPS production by *Rhizobium* strain was achieved by optimizing the cultural conditions (Carbon and Nitrogen sources). The EPS production was maximum, when the medium was supplemented with mannitol (2%) and potassium nitrate (0.2%) which was accompanied by a great increase in the production compared to the control. The EPS contained glucose, galactose, xylose, rhamnose and raffinose, which were identified by paper chromatography.

Key words: Extracellular Polysaccharides, *Vigna mungo*, *Rhizobium*, Carbon source, Nitrogen source.

INTRODUCTION

A Leguminous plant is important both ecologically and agriculturally, since it is a major source of biological nitrogen fixation through root nodules formation. Root nodules are a unique and highly organized structure developed on a result of the symbiotic relationship between leguminous plants and bacteria of the genus *Rhizobium*. 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.* [1] reported about the enhancement of nodulation by EPS. *Rhizobium* species were described which were able to produce EPS also in culture [2, 3]. Skorupska *et al.* [4] also reported that extracellular polysaccharides may be involved in invasion and nodule development, bacterial release from infection threads, bacteriod development, suppression of plant defense response and protection against plant antimicrobial compounds.

Exopolymer production by bacteria has generated increasing attention among researchers for the last few years, because of its commercial interest. The exopolymer include a diverse range of molecules that play vital role in a variety of biological processes [5]. The EPS is economically important because it can impart functional effects to foods and confer beneficial health effects [6]. Now-a-days, a lot of effort is put into the selection of new microbial strains and optimization of culture conditions to achieve higher yields of those EPSs already commercially successful. Furthermore, there is a considerable interest in finding new EPSs that are suitable for special applications, or that have potential industrial relevance, either by applying different culture conditions or by using novel bacterial strains [7]. EPS characteristics and amounts can be mostly influenced by the composition of the medium (carbon and nitrogen sources) and its concentration [8].

Vigna mungo L. (Hepper) is an economic leguminous plant (Fabaceae). It is an erect, sub erect or trailing, densely hairy annual herb. The tap root produces a branched root system with smooth, rounded nodules. Little information has been gathered about its nodules and their symbionts.

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The objective of this study was to screen the maximum EPS producing strain from *V. mungo* and also to increase the production of EPS through optimization of cultural conditions (carbon and nitrogen sources) of the strain, which produced maximum amount of EPS.

MATERIALS AND METHODS

Chemicals, media and media components

All the chemicals, media and media components used were of analytical grade obtained from Himedia Laboratories Ltd, India.

Isolation and screening of organism for EPS production

A total of ten *Rhizobium* strains were isolated from fresh healthy root nodules of *Vigna mungo* collected from different regions of Chennai, India. For isolation of bacteria, healthy pink, unbroken, firm root nodules were selected, surface sterilized, crushed and streaked on Yeast Extract Mannitol Agar medium(YEMA) and incubated at 30 ± 2º C for 3-4 days. The isolated colonies were purified and stored. These strains were identified as species of *Rhizobium* following Bergey's Manual of Systematic Bacteriology [9].

The basal medium for the bacterial growth and EPS production was the yeast extract mineral medium [10] with 1% mannitol at pH 6.9. For the preparation of the inoculum, a loopful of the bacteria from a slant was grown in 25 ml of medium for 12h. The optimum inoculum dose used for each experiment was 2ml.

For determining maximum EPS producing ability of the isolates, the bacterial strains were incubated in 25ml of the medium in 100ml conical flasks in three replicates at 30 ± 2º for 72h (optimum time for maximum EPS production) [11]. The growth was measured spectrophotometrically at 610 nm and the EPS was estimated by Phenol-Sulphuric acid method [12]. The isolate which produced maximum amounts of EPS was carried out for further tests.

Production of EPS on different sources

All the supplements added to the medium were filter sterilized and added to the medium aseptically.

Effect of carbon and nitrogen sources on EPS production

Different carbon sources (1% each) such as glucose, galactose, fructose, lactose, maltose, mannitol, mannose and sucrose were added individually to the basal (YEM) medium replacing mannitol. The medium was inoculated with maximum EPS producing *Rhizobium* isolate and grown at 30 ± 2º C for 72h. Then the EPS was isolated and estimated.

Different nitrogen sources (0.1% each) such as ammonium sulphate, sodium nitrate, potassium nitrate, glycine and glutamic acid were added separately to the basal (YEM) medium containing the most suitable carbon source. The medium was inoculated with *Rhizobium* isolate and grown at 30 ± 2º C for 72h. Then the EPS was isolated and estimated.

Effect of different concentrations of most suitable carbon and nitrogen source

The carbon source which showed maximum EPS production was varied in its concentration (1% to 4%) and added to growth medium to assess the effect of carbon concentrations on EPS production by *Rhizobium* isolate.

The nitrogen source which showed maximum EPS production was varied in its concentration (0.05% to 0.20%) and added to growth medium to assess the effect of nitrogen concentrations on EPS production by *Rhizobium* isolate.

Thus the individual effect of these chemicals on EPS production was measured.

Maximum EPS production by *Rhizobium* isolate

For maximum EPS production by the isolate, the medium was enriched with carbon and nitrogen supplements which individually increase the EPS production to maximum level.

Isolation of EPS

Isolation of EPS was done by following the method described by Dudman [13] and collected by centrifugation at 6000xg for 20 min, dissolved in minimum volume of distilled water, reprecipitated with 3 volumes of acetone, centrifuged at 6000xg for 20 min, resuspended in distilled water, dialyzed and lyophilized.

For the identification of sugar monomers, dry polysaccharides were hydrolyzed in sealed culture tubes with 0.5 M H_2SO_4 at 100^oC for 16h, neutralized by $BaCo_3$ and filtered. The filtrate was concentrated at 45^oC under reduced pressure. The monosaccharides were chromatographed on WHATMAN paper No.1 using the solvent system butanol: acetic acid: water (4:3:1, v/v/v) according to Heftman [14]. The sugar components were identified by the aniline phthalate spraying reagent [15].

Estimation of EPS

The dialyzed cell free supernatant was used for EPS estimation using phenol-sulphuric acid

method following Dubios *et al*., [12] using glucose as the standard [16].

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 *Vigna mungo* were designated as *Rhizobium* Vm1 to Vm10. The isolated strains were identified as species of *Rhizobium* following Bergey's Manual of Systematic Bacteriology [9]. Among the 10 *Rhizobium* strains tested, the *Rhizobium* Vm6 produced maximum amount of EPS on Yeast Extract mineral medium (**Table 1**). As *Rhizobium* Vm6 produced more amounts of EPS, further tests were carried out on this strain.

The EPS synthesis by microbial cells depends on the carbon and nitrogen availability in the culture medium. Most exopolymer-producing microorganisms utilize carbohydrates as their carbon and energy source and either ammonium salts and amino acids as their source of nitrogen [17, 18]. Thus these sources (type and its concentration) have a huge influence on EPS productivity.

All the eight tested carbon sources (1%) enhanced both bacterial growth and EPS production to different extents. Among them, mannitol was the most suitable promoter followed by sucrose (**Table 2**). This upholds the views of Ghosh *et al*., [3] and Prabhavathi and Mallaiah [11]. Both of them reported that mannitol was the best carbon source in *Rhizobium* DL10 species from *Dalbergia lanceolaria* and HGR12 from *Macrotyloma uniflorum*. Cigdem Cucuk and Merih Kivanc [20] also reported that mannitol was the efficient carbon source in EPS production by *Rhizobium ciceri* Rc5 from Turkey. Breedveld *et al*., [20] also reported about the utilization of mannitol by *Rhizobium leguminousarum* for EPS production. But Sridevi and Mallaiah [21] reported that galactose was the best carbon source for EPS production by *Rhizobium* SS5 from *Sesbania sesban*.

Among the nitrogen sources tested, maximum EPS production was observed in potassium nitrate followed by sodium nitrate (**Table 3**). The present result was supported by earlier works done by Ghosh *et al.*, [3] and Datta and Basu [22]. They reported that potassium nitrate is the best nitrogen source for EPS production in *Rhizobium* DL10 from *Dalbergia lanceolaria* and *Rhizobium* from *Cajanus cajan*. But Sridevi and Mallaiah [21] reported that sodium nitrate is the best nitrogen source for *Rhizobium* SS5 from *Sesbania sesban*. Cigdem Cucuk and Merih Kivanc [19] also reported that sodium nitrate is the best nitrogen source for *Rhizobium ciceri* from Turkey. Prabhavathi and Mallaiah [11] reported that ammonium sulphate is the best nitrogen source for *Rhizobium* HGR12 from *Macrotyloma uniflorum*. Ghosh and Basu [23] reported that casaminoacids promoted both growth and EPS production.

The optimum concentration of mannitol required for EPS production was found to be 2%. The optimum concentration of potassium nitrate was found to be 0.2% (**Table 4**).

To test the maximum EPS production by *Rhizobium* Vm6 strain in culture, the supplements which individually increased the production to the greater extent was added to the medium. The bacteria which initially produced 1680 µg/ml EPS in basal Yeast extract mineral medium was induced to produce a greater amount of EPS through optimization (**Table 5**).

From this result, we conclude that the EPS synthesis by microbial cells depends on carbon and nitrogen availability in the culture medium and the most suitable carbon and nitrogen source which enhances the EPS production is mainly dependent on species specific of *Rhizobium* species nodulating different leguminous plants.

The sugar monomers which were present in EPS were identified as glucose, galactose, xylose, rhamnose and raffinose by paper chromatography. The present results support the views of Prabhavathi and Mallaiah [11] and Sridevi and Mallaiah [21]. But Ghosh *et al.*, [3] reported that EPS produced by *Rhizobium* DL10 species contained glucose, galactose, xylose, rhamnose and arabinose. Hollingsworth *et al*., [24] also observed the presence of glucose, galactose, and mannose in EPS, which was secreted by *Rhizobium* strain of M1-50A, M6-78 and IRC 253 of cowpea rhizobia. EPS of some members of Rhizobiaceae contain mannitol and fructose [20]. Some strains of *Bradyrhizobium japonicum* contained EPS having rhamnose and 4-0-methylglucuronic acid [25]. These have indicated that there are variations in the sugar monomers from different *Rhizobium* species.

Correlation between the growth and EPS production in YEM medium is positive (r= 0.31). The effect of carbon and nitrogen source also

showed positive correlation that of nitrogen sources is highly positive (r=0.88).

Table 1: Production of Extracellular Polysaccharides (Eps) By Rhizobium Strains from *Vigna mungo*.

Rhizobium isolates(Vm)	Growth (OD at 610nm)	EPS production (µg/mL)
Vm1	1.375	1050
Vm2	0.468	980
Vm3	0.770	1420
Vm4	0.680	1074
Vm5	0.580	1030
Vm6	0.910	1680
Vm7	0.414	920
Vm8	0.739	1080
Vm9	0.392	1090
Vm10	0.608	1280

Correlation coefficient between growth and Extracellular Polysaccharide production (r= 0.31).

Table 2: Effect of Different Carbon Sources on Growth and Extracellular Polysaccharide Production by *Rhizobium* Vm6.

Carbon sources	Growth (OD at 610nm)	EPS production (µg/mL)
Control*	0.121	180
Glucose	0.692	1260
Galactose	0.343	530
Fructose	0.853	255
Lactose	1.402	561
Mannose	1.700	714
Mannitol	0.838	1530
Maltose	0.742	1020
Sucrose	0.756	1360

*The control medium was devoid of any additional carbon source; Correlation coefficient between growth and Extracellular Polysaccharide production (r = 0.12)

Table 3: Effect of Different Nitrogen Sources on Growth and Extra Polysaccharide Production By *Rhizobium* vm6.

Nitrogen sources	Growth (OD at 610nm)	EPS production (µg/mL)
Control*	0.160	153
Ammonium sulphate	0.166	224
Glycine	1.509	200
Glutamic acid	3.00	510
Potassium nitrate	4.29	1680
Sodium nitrate	4.00	1071

*The control medium was devoid of any additional nitrogen source; Correlation coefficient between growth and Extracellular Polysaccharide production (r = 0.88)

Table 4: Effect of Different Concentrations of Mannitol and Potassium Nitrate on Growth and Extracellular Polysaccharide Production by *Rhizobium* Vm6.

Concentrations (%)	Growth (OD at 610nm)	EPS production (µg/mL)
Mannitol concentration		
1.0	0.82	1500
2.0	0.86	1570
3.0	0.41	670
4.0	0.30	450
Potassium nitrate		
0.05	0.24	300
0.10	0.21	260
0.15	0.18	200
0.20	0.28	400

Table 5: Increase in Growth and Extracellular Polysaccharide Production Using *Rhizobium* Vm6 with the Most Effective Supplements

Supplements	Growth (OD at 610nm)	EPS production (µg/mL)
Control	0.910	1680
Mannitol + Potassium nitrate	4.402	3426

In the control set, the bacteria were grown in yeast extract mineral medium. In other cases, the medium was supplemented with mannitol (2%) and potassium nitrate (0.2%)

CONCLUSION

All the supplements which increased the EPS production in culture could be available for the *Rhizobium* 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 (10). Moreover, the increased EPS production by the strain Vm6 could be useful for the industry.

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