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Extracellular polysaccharide production by *Rhizobium* sp. nodulating *Macrotyloma uniflorum* (Lam.) Verdc

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The ability of thirty two *Rhizobium* strains, isolated from root nodules of *Macrotyloma uniflorum* (Lam.) Verdc. were tested for their production of extracellular polysaccharides (EPS) in Yeast Extract Mannitol (YEM) medium. Among the thirty two isolates, maximum amount of EPS was produced by the isolate HGR12 (4690 μg mL^{-1}). Both growth and EPS production started simultaneously. The production of EPS was maximum in the stationary phase of growth (72 hr) when the medium was supplemented with mannitol (1%). The EPS contained glucose, galactose, xylose, rhamnose and raffinose, which were identified by paper chromatography.

Key words : Extracellular polysaccharides, *Rhizobium* sp., *Macrotyloma uniflorum*, Legume – *Rhizobium* association.

INTRODUCTION

The production of rhizobial extracellular polysaccharides (EPS) has created great interest among scientists for a long period of time. Rhizobial EPS act as determinants of host plant specificities and play a role in the initial step of root hair infection (Olivares *et al.*, 1984). EPS protects the organism from the adverse environmental condition(s).

Macrotyloma uniflorum (Lam.) Verdc. is an important pulse and green manure crop. Thirty two *Rhizobium* strains were isolated from the root nodules of *M. uniflorum* plants growing in thirty two soil samples collected from various parts in Andhra Pradesh. Very little information is available on cultural characters of rhizobia associated with this pulse crop. Hence the present work was taken up to study the factors effecting EPS production by the *Rhizobium* sp. isolated from *M. uniflorum*.

MATERIALS AND METHODS

Medium and growth conditions:

The basal medium for the bacterial growth and EPS production was the yeast extract mineral medium (Skernan, 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 72 h (optimum time for maximum EPS production). The growth was measured spectrophotometrically at 610 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 isolate, 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 re-precipitated with 3 volumes of acetone, centrifuged, dialyzed and lyophilized. For identification of sugar monomers, dry EPS was hydrolyzed in a sealed tube with 0.5M $BaCO_3$ 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 pthalate (Patridge, 1949). The sugar derivatives were identified by comparison 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). The data were statistically analyzed using correlation coefficient between growth and EPS production.

RESULTS AND DISCUSSION

The *Rhizobium* isolates nodulating *M. uniflorum* were

fast growers and reached stationary phase by 72 hr. Among the thirty two *Rhizobium* isolates tested, the isolate HGR12 produced maximum amount of EPS on yeast extract mannitol (YEM) broth (Table 1) maximum EPS production was also observed at 72 hr by this isolate. As HGR12 produced more amounts of EPS, further tests were carried out on this isolate.

Of the fifteen carbon sources (1 %) tested for their effect on growth and EPS production, maximum amount was observed in mannitol, followed by lactose (Table 2).

Table1 : Production of extracellular polysaccharides (EPS) by *Rhizobium* isolates from *M. uniflorum*

Rhizobium Isolates (HGR)	Growth OD at 610 nm	EPS production μg mL^{-1}	Specific productivity EPS production/growth
1	1.365	1040	1713.0
2	0.238	1060	989.1
3	0.454	960	1128.2
4	0.862	1090	1390.1
5	0.485	1070	1148.1
6	0.775	1430	1334.3
7	0.780	1130	1337.5
8	0.660	1050	1260.5
9	0.469	1020	1137.9
10	1.182	1020	1595.6
11	0.590	1040	1215.5
12	1.114	4690	1551.9
13	0.708	870	1291.3
14	0.720	1180	1299.6
15	0.845	1220	1379.2
16	0.557	1980	1194.4
17	0.495	1180	1154.6
18	0.484	1460	1147.5
19	1.121	1000	1556.4
20	0.900	1640	1414.5
21	0.727	990	1303.5
22	0.414	950	1102.6
23	0.716	1340	1296.4
24	0.853	1100	1384.4
25	0.729	1070	1304.8
26	0.555	1560	1193.1
27	0.918	1100	1426.1
28	0.720	1200	1299.0
29	0.505	1420	1161.0
30	0.598	1270	1220.7
31	0.382	1080	1082.0
32	0.690	1084	1279.7

*Correlation coefficient between growth and extracellular polysaccharide production (r = 0.24)

Table 2 : Effect of different carbon sources on growth and extracellular polysaccharide production by the isolate HGR12

Carbon source	Growth OD at 610 nm	EPS production μg mL^{-1}	Specific productivity (EPS production/growth)
Control	0.524	450	487.0
Mannitol	1.114	4690	4513.0
Glucose	0.580	2430	2201.2
Galactose	0.240	1170	729.3
Raffinose	0.286	1124	928.4
Xylose	0.182	1140	478.2
Inositol	0.342	628	1170.9
Fructose	0.532	700	1993.4
Lactose	0.537	3110	2015.1
Maltose	0.348	840	1196.8
Sucrose	0.230	420	686.0
Ribose	0.220	1100	642.7
Trehalose	0.242	606	737.9
Arabinose	0.360	1100	1248.8
Mannose	0.302	482	997.1

*Correlation coefficient between growth and extracellular polysaccharide production (r = 0.81).

Mannitol was the best carbon source was reported earlier in *Rhizobium* D110 sp. from *Dalbergia lanceolaria* (Ghosh *et al.*, 2005). The optimum concentration of mannitol required for maximum EPS production was found to be 1% (Table 3).

Among the nitrogen sources tested, maximum EPS production observed in ammonium sulphate followed by sodium nitrate (Table 4), although maximum growth was observed in casamino acid. But, Ghosh and Basu (2001) reported that casamino acids promoted both growth and EPS production.

Thiamine was effective source for maximum EPS

Table 3 : Effect of different concentrations of mannitol and ammonium sulphate on growth and extracellular polysaccharide production by the strain HGR12

Concentrations (%)	Growth (OD at 610 nm)	EPS Production (μg mL^{-1})
Mannitol concentration		
1.0	1.114	4690
2.0	1.108	3820
3.0	1.100	3426
Ammonium sulphate		
0.05	0.250	720
0.10	0.268	760
0.15	0.262	722
0.20	0.230	690

Table 4 : Effect of different nitrogen sources and vitamins on growth and extracellular polysaccharide production by the isolate HGR12

Sources	Growth (OD at 610 nm)	EPS production (μg mL^{-1})	Specific productivity (EPS production/ growth)
Control	0.015	120	466.7
Ammonium sulphate	0.268	760	540.5
Potassium nitrate	0.208	662	523.0
Sodium nitrate	0.164	668	510.2
Glycine	0.151	650	506.4
Glutamine	0.148	530	505.5
Glutamic acid	0.237	550	531.5
L-aspargine	0.130	060	500.3
Alanine	0.119	630	497.1
Tyrosine	0.418	660	584.3
Casamino acid	1.034	640	764.0
Vitamins			
Control	1.114	4690	2201.2
Riboflavin	0.263	668	651.2
Thiamine HCl	0.174	720	556.9
Nicotinic acid	0.158	520	540.0
Ascorbic acid	0.168	520	550.6
Pyridoxal phosphate	0.184	480	567.5
Ca-pantothenate	0.201	544	585.5

* Control was devoid of any type of additional and vitamin sources; Correlation coefficient between growth and extracellular polysaccharide production in nitrogen sources (r = 0.22), in vitamins (r = 0.42).

Table 5 : Effect of metal chlorides and cell wall affecting agents on growth and extracellular polysaccharide production by the isolate HGR12

Sources	Growth (OD at 610 nm)	EPS production (μg mL^{-1})	Specific productivity (EPS production/growth)
Metals			
Arsenic Chloride	0.212	420	534.2
Calcium Chloride	0.250	460	519.0
Copper Chloride	0.253	590	517.8
Mercuric Chloride	0.157	530	556.1
Ferric Chloride	0.277	550	508.2
Magnesium Chloride	0.220	540	531.0
Manganese Chloride	0.138	620	563.7
Stannous Chloride	0.173	570	549.7
Cell wall affecting agents			
Control	1.114	4690	4630.7
EDTA	0.184	540	803.0
Penicillin	0.162	424	712.5
SDS	0.086	480	399.7
Lysozyme	0.040	400	210.4
Tween 80	0.066	540	317.4

*Correlation coefficient between growth and extracellular polysaccharide production in cell wall affecting agentsis highly positive (r = 0.99).

production at 1 μg mL^{-1}. Ghosh and Basu (2001) reported that biotin at 1 μg mL^{-1} increased both growth and EPS production in Azorhizobium caulinodans.

Among the metals maximum EPS (Table 5) was observed in copper chloride containing medium. Ghosh and Basu (2001) reported that maximum growth and EPS production were found at 10 μg mL^{-1} of manganous chloride. Different cell wall/ membrane affecting agents had no significant effect on both growth and EPS production of this isolate.

The EPS produced by the isolate contained glucose, galactose, xylose, rhamnose and raffinose, which were identified by paper chromatography. Hollingsworth *et al*. (1985) also observed the presence of glucose, galactose and mannose in EPS, which were secreted by cowpea rhizobia. EPS of some members of Rhizobiaceae contains mannitol and fructose (Breedveld *et al.*, 1993). These studies indicated that there are variations in the sugar monomers from different *Rhizobium* sp.

The EPS secreted by this isolate was acidic indicating the presence of uronic acid. The amount of uronic acid was found to be 348.2 μg mL^{-1} of EPS. Amemura *et al*. (1983) have reported that most extracellular acidic polysaccharides of *R.. trifolii* contained D-glucouronic acid.

Correlation between the growth and EPS production in YEM medium is positive (r = 0.24). The effect of carbon, nitrogen, vitamins, metals and cell wall/membrane affecting agents also showed positive correlation that of cell wall/membrane affecting agents is highly positive (r = 0.99).

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