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Extracellular polysaccharide production by *Azorhizobium caulinodans* from stem nodules of leguminous emergent hydrophyte *Aeschynomene aspera*

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The *Azorhizobium caulinodans* isolated from the stem nodules of a leguminous emergent hydrophyte, *Aeschynomene aspera*, produced a large amount of extracellular polysaccharides (EPS) in yeast extract basal medium. Maximum EPS production was at the stationary phase of growth. EPS production was increased by 919% over control when the medium was supplemented with sucrose (1.5%), D-biotin (1 µg /ml) and casamino acid (0.1%). EPS contained rhamnose and arabinose. Possible role of the azorhizobial EPS production in the stem nodule symbiosis is discussed.

Root nodules of leguminous plants created a great interest among scientists. Many aspects of the symbiosis in the root or stem nodules, such as nitrogen fixation and hormone production have been extensively investigated. But some aspects of the symbiosis like extracellular polysaccharide (EPS) production are still poorly understood. *Rhizobium* spp. produce large amounts of EPS which were thought to act as determinants of host plant specificity and to play a role in the first steps of root hair infection (see Olivares *et al.*)[1]. Addition of crude EPS preparations from highly infective *R. meliloti* increased the infectivity of a poorly infective strain[1]. *Aeschynomene aspera* is a less explored leguminous shrub having enormous economic importance. Stems of the plants are used for making fishing floats, swimming jackets, sunhats, bottle cork, paper etc. The pith is used as a substitute for surgical lint. It has high insulating properties. The seeds yield an oil[2]. The plant bears large numbers of sessile stem nodules, which contain high amount of IAA[3]. The symbiont of the stem nodules also produced large amount of IAA in in culture[4]. The purpose of this study was to check the ability of *Azorhizobium caulinodans* isolated from the stem nodules of this leguminous emergent hydrophyte for EPS production for its importance in nodule symbiosis and in industry.

Materials and Methods

Microorganism, medium and growth conditions—The symbiont was isolated from fresh healthy stem he symbiont was isolated from fresh healthy stem nodules of *Aeschynomene aspera* and was grown in axenic culture. The basal medium for the bacterial growth was the yeast extract mineral medium of Skerman[5] with 1% mannitol, containing 0.01 % $CaCl_2$. $2H_2O$ instead of NaCl and $CaCO_3$ at *p*H 7.0. The bacteria were incubated with three replicates at $30° \pm 2°$ C on a rotary shaker for 40 hr (optimum time for maximum EPS production). The bacterial growth was measured spectrophotometrically at 540 nm and also by taking cell count in a Neubauer counting chamber (data not shown).

Culture of the symbiont—Different carbon sources were added separately to the basal medium omitting mannitol. Individual effect of different chemicals with most suitable carbon source on EPS production was checked. For maximum EPS production by the symbiont in culture, the medium was enriched with the supplements which individually increased the EPS production to the maximum level. All supplements added to the medium for cultural optimization were filter sterilized.

Isolation of extracellular polysaccharides—EPS produced was precipitated after growth from the culture filtrate following Dudman[6] and collected by centrifugation, dissolved in a minimum volume of distilled water, reprecipitated with three volumes of acetone, centrifuged, dialyzed and lyophilized.

For identification of sugar monomers, dry EPS was hydrolyzed in a sealed tube with 0.5 *M* of H_2SO_4 at 100°C for 16 hr, neutralized by $BaCO_3$ and concen-

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trated 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[7]. For gas liquid chromatographic analysis, sugar derivatives (peracetylated alditols) were prepared from dry lyophilized polysaccharides following Lindahal[8] and injected in GLC apparatus (J and W fused silica capillary column, oven temperature 230°C, carrier gas H_2 3-4 mL/min). 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-sulfuric acid method following Dubois[9]. Uronic acid estimation in EPS was performed by carbazole reaction following Dische[10]. The statistical analyses were done following Panse and Sukhatme[11] taking 3-5 replicates.

Results and Discussion

The symbiont, isolated aseptically from the stem nodules of *A.. aspera,* was identified following Conn *et al.*[12] and Jordan[13] to be a fast growing species of *Rhizobium* (data not shown). However, using DNA-DNA hybridization, total protein profile analyses and other methods, Dreyfus *et al.* have assigned the symbionts of stem nodules to a new genus and species, *Azorhizobium caulinodans*[14]. We have adopted this nomenclature[4]. *A. caulinodans* reached its stationary phase of growth after 16 hr in yeast extract mannitol (YEM) medium. Both bacterial growth and EPS production were started simultaneously (Fig. 1) and maximum EPS production was found after 40 hr, much later than the growth has reached the stationary phase (16 hr). Caviedes *et al.*[15] have reported similar increase in EPS production during the stationary phase of cultures of *Rhizobium trifolii* strains.

All the carbon sources tested (1 %) promoted both bacterial growth and EPS production to a different extent (Table 1). Sucrose was the best promoter , of which 1 .5 % was most effective (Fig. 2). Not all the inorganic and organic nitrogen sources tested were effective in increasing growth or EPS production (Table 2). Casamino acids (0.1 %) promoted both growth and EPS production to the maximum (Fig. 3). The vitamines had little promotive effect on growth (Table 3). But 1 µg /mL of D-biotin increased both growth and EPS production. Different metal ions and cell wall / membrane affecting chemicals had no significant effect on both growth and EPS production by the symbiont. The bacteria which initially produced 36 µg /mL of EPS in carbon free yeast extract mineral medium were stimulated to produce 367 µg / mL of EPS which were an enormous increase (919 % over control) through cultural optimization (Table 4).

Sugar monomers in EPS produced by this *A. caulinodans* were identified by paper chromatography and gas liquid chromatography (GLC) and detected as

Table 1—Effects of different carbon sources on growth and EPS production in culture by *A. caulinodans.*

Carbon sources	Growth (OD at 540 nm)	EPS production (µg /mL)
Control	0.74	41
Myo-Inositol	1.35	184
Raffinose	1.40	194
Lactose	1.50	194
D(+) Mannose	1.55	194
D (-) Mannitol	1.48	199
D (+) Galactose	1.55	199
Glucitol	1.45	204
D-Fructose	1.50	204
D-Glucose	148	214
L (+) Arabinose	1.65	214
D-Ribose	1.50	219
Maltose	1.55	224
D-xylose	1.55	230
Sucrose	1.62	235
Critical difference at P = 0.05	0.03	10

The bacteria were grown in yeast extract mineral medium for 40 l at 30° ± 2°C. Carbon sources were supplemented individually at 1% level except in control set .

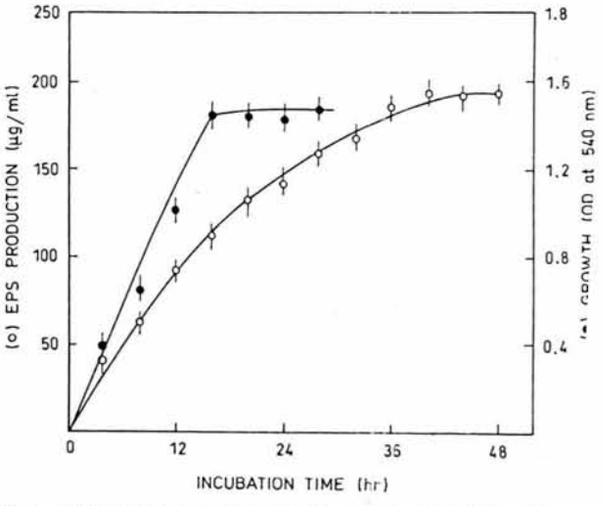

Fig.1—Extracellular polysaccharide production (o) and gr (●) by *A. caulinodans* in culture. The bacteria were grow YEM medium at 30° ± 2° C. Bars at the points indicate ± SE.

80.78% rhamnose and 19.22% arabinose taking total amount of the two sugars as 100%. Huber *et al.*[16] have found some strains of *Rhizobium japonicum* to contain EPS having rhamnose and 4-0-methylglucuronic acid. Hollingsworth *et al.*[17] have reported the presence of glucose, galactose and mannose in polysaccharides secreted by *Rhizobium* strains MI-50A, M6-7B and IRC 253. Some *Rhizobium* spp. have been found to contain EPS having mannitol and glucitol[18]. EPS of some members of Rhizobiaceae contains mannitol and fructose[15], glucans and cellulose[19]. These have indicated that there are variations in the composition of EPS from different *Rhizobium* spp. EPS secreted by this symbiont also contains uronic acid. The amount of uronic acid is 221.1 mg/ g of EPS. Amemura *et al.*[20] have reported that most extracellular acidic polysaccharides of *Rhizobium trifolii* contained D-glucuronic acid.

All the supplements which increased the EPS production in culture might be available to the symbiont within the stem nodules. These might further stimulate the bacteria for more polysaccharide production which might increase the infection. The nodulation

Table 2—Effects of different nitrogen sources on growth and EPS production in culture by *A. caulinodans*

Nitrogen sources	Growth(OD at 540 nm)	EPS production (μg/ml)
Control	1.74	291
$(NH_4)_2SO_4$	1.24	219
$NaNO_3$	1.12	230
KNO_3	1.32	230
NH_4Cl	1.08	235
Glycine	1.60	255
L-Glutamic acid	3.30	342
L-Asparagine	3.60	342
Casamino acid	4.50	357
Critical difference at P= 0.05	0.06	12

The bacteria were grown in yeast extract mineral medium with 1.5 % sucrose . The nitrogen sources were supplemented at 0.1 % level individually except in control set. Other conditions were same as in Table 1.

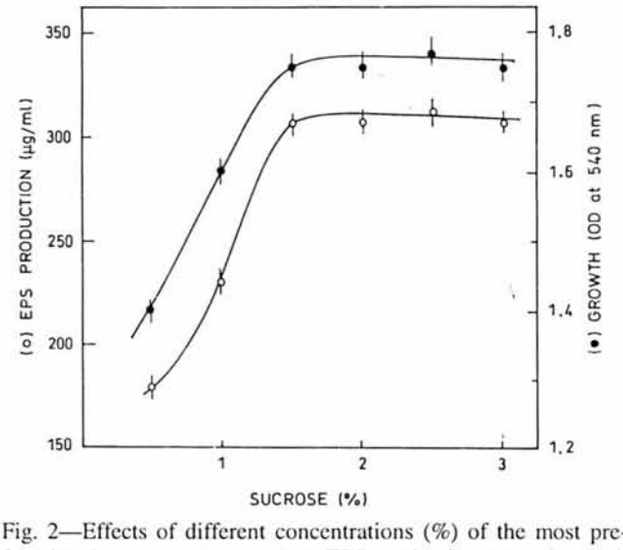

Fig. 2—Effects of different concentrations (%) of the most preferred carbon source (sucrose) on EPS production (o) and growth (●) in culture by *A . caulinodans* . The bacteria were grown for 40 hr. Other conditions were same as in Fig. 1. Bars at the points indicate ± SE.

Table 3—Effects of different vitamins on growth and EPS production by *A caulinodans.*

Vitamins	Growth (OD at 540 nm)	EPS production (μg/ ml)
Control	1.80	296
Riboflavin	1.76	291
Thiamine- HCl	1.84	296
Nicotinic acid	1.76	306
Pyridoxal phosphate	1.88	306
Ca-pantothenate	1.88	311
Ascorbic acid	1.82	321
D-Biotin	1.90	347
Critical difference at P =0.05	0.04	9

The bacteria were grown in yeast extract mineral medium with 1.5 % sucrose. Vitamins were supplemented at 1 μg/mL level individually except in control set. Other conditions were same as in Table 1.

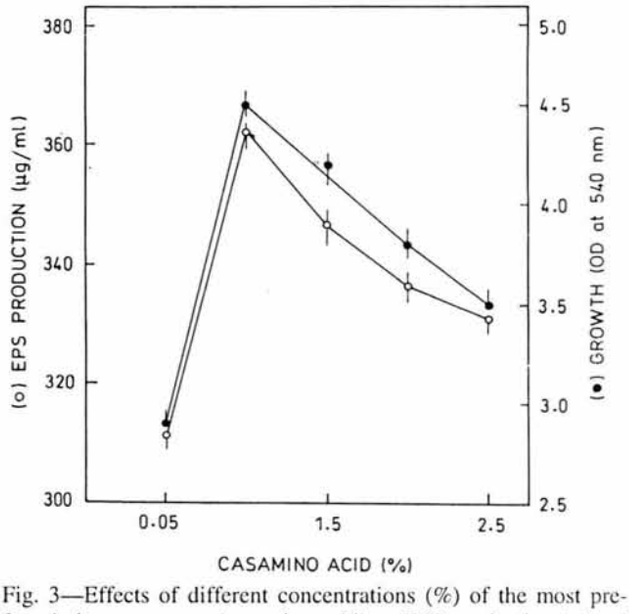

Fig. 3—Effects of different concentrations (%) of the most preferred nitrogen source (casamino acid) on EPS production (o) and growth (●) in culture by *A. caulinodans* at 30° ±2°C. Other conditions were same as in Fig. 2 .Bars at the points indicate ± SE

Table 4—Increase in growth and EPS production by *A. caulinodans* with the most effective supplements

Supplements	Growth		EPS production	
	(OD at 540 nm)	(%increase over control)	(μg/mL)	(%increase over control)
Control	0.70	—	36	—
+ Sucrose	1.80	157	306	750
+Sucrose + D-Biotin	1.92	174	337	836
+Sucrose +D-Biotin +Casamino acid	4.56	551	367	919

In control set the bacteria were grown in a carbon free yeast extract mineral medium . In others, the medium was supplemented with sucrose (1.5%), D-Biotin (1μg/ml) and casimino acids (0.1%). Other conditons were same as in Table 1.

rate has been shown to be increased after addition of specific crude EPS (ref. 1). Caviedes *et al.*[15] have found that EPS is required for nodulation by *Rhizobium trifolii*. Rhizobial exopolysaccharides act as determinants of host plant specificities[1,21] and play a role in the initial step of root hair infection[1,22]. Mutants deficient in exopolysaccharide production (exo$^-$) could not induce nitrogen fixing nodules[23]. But some exo$^-$ mutants are able to induce nitrogen fixing nodules on clover because of the presence of added homologous EPS from wild type strains[23]. In addition to the functions in symbiosis, the increased EPS production in culture by this symbiont might be helpful in industries because some bacterial polysaccharides have importance in food, pharmaceutical and cosmetic industries to form gels, colloids, creams etc.[19,24]. The widest non-food industrial application of bacterial polysaccharides is the use of xanthan in the oil industry.[25] The significant biomedical exploitation of bacterial polysaccharides had been use as vaccine agents[24]. Thus, the increased EPS production by *A.. caulinodans* might have dual benefit, in increased infection leading to nodulation and in industry.

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