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Effect of different carbon, nitrogen and vitamine sources on exopolysaccharide production of *Rhizobium* species isolated from root nodule of redgram

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Three bacterial strains CHRS-7, RAB-1 and RAN-1 were isolated from root nodules of redgram (*Cajanus cajan*) and identified as *Rhizobium* sp. based on 16S rDNA sequence homology and assigned accession number MH636329, MH636773 and MH541051, respectively by National Center for Biotechnology Information (NCBI). All the strains could produce copious amount of exopolysaccharides in yeast extract manitol broth medium. All the three strains had different stationary phases but the bacterial growth and exopolysaccharides production occurred simultaneously. The glucose (1.5 %), manitol (2.0%) and sucrose (1.5%) were the preferable carbon sources of CHRS-7, RAB-1 and RAN-1 respectively, for both growth and EPS production. Among the nitrogen sources glycine (0.1%), $NaNO_3$ (0.1%) and KNO_3 (0.1%) were the preferable N sources for CHRS-7, RAB-1 and RAN-1 respectively, whereas, CHRS-7, RAB-1 and RAN-1 preferred biotin 1.5%, 2.0% and 1%, respectively.

Keywords: *Cajanus cajan*, EPS, *Rhizobium*, Root nodule

Most of the bacterial species produce exopolysaccharides and are transported to the extracellular space and it will exist as either soluble or insoluble forms. Exopolysaccharides may loosely attach to the cell surface or completely excreted into environment as slime[1,2].

The N-fixing symbiotic bacteria comprise very diverse group of gram-negative soil bacteria (α and β-proteobacteria) collectively called rhizobia, is a unique feature of the plants belonging to legume family[3] and have the ability to produce root nodule by symbiosis with legume plants. The symbiosis is a complex process involving a coordinate exchange of signal between legume plants and symbiont[4] and it depends on the production of bacterial exopolysaccharide and nod factor signal[5].

Rhizobia are well-known for production of copious amount of EPS into the rhizosphere as well as in pure cultures[6] and increase the viscosity in culture medium. The EPS production and biofilm formation by bacteria has better effect on soil fertility and plant growth[2].

The biofilms are held together by an extracellular polymeric substances (compounds that forms outside the cell wall) or EPS. The EPS are held together and developed in to a complex, that are resistant to stress. The EPS consists of branched or repeating units of sugars/sugar derivatives and other non-carbohydrate components[7-9]. It is a new and industrially important source of polymeric material[10]. The extra cellular polysaccharides covers the biofilm are usually hygroscopic, therefore microorganisms create more hydrated micro-environment in the immediate vicinity of cells causes desiccation tolerance[11]. The exploitation of exopolysaccharide producing microorganisms has greatly increased in recent years, since these biopolymers attracted worldwide attention because of their unique physical properties as bioflocculants, stabilizers, biosorbents, bioadhesives, gelling agents, probiotics, and thickeners. The EPS producing microorganisms has made them suitable for commercial applications in the pharmaceuticals, food industry, cosmetics, environmental sectors, civil construction, petroleum and bionanotechnology. These are eco-friendly, non-toxic, and biodegradable[1,12].

The exopolysaccharide (EPS) is the biopolymers found attached to the surface of cells or secreted into the extracellular medium[13]. The EPS producing different legume nodulating (particularly α-proteobacteria) bacteria[14-18] are capable of forming gels at low concentrations[19,20]. These EPSs produced by symbiont

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are considered as signaling molecules, which is essential for the formation of infection thread, successful root tissue invasion and initiation of nodule formation[4]. It is also one of the signals required for determining host plant specificity during the early stage of root hair infection[21]. These EPSs have also mechanistic role to provide protection against moisture stress[9,22]; abiotic stress[23,24]; predation, bacteriophages, antimicrobial compounds and attachment to surfaces[25]; chelating various metal ions and promoting the growth of plant is well established. It serves as a potential energy reserve as it can be catabolized under nutrient deficient conditions[22].

Large number of microorganisms including *Rhizobium* sp.[26-28] produce different types of exopolysaccharide. The synthesis of rhizobial EPS has been best studied in two species *i.e. Sinorhizobium meliloti* and *Rhizobium leguminosarum*[29]. Influence of culture conditions on polysaccharide production are reported for various organisms[26-28,30]. The Rhizobia produced large amount of EPS in culture rather than EPS produced in symbiotic condition[31]. The *Azorhizobium caulinodans* ORS 571-*Aspergillus* spp. produces biofilm in the presence of naringen in rhizosphere of rice[11]. The growth environment was very important for maximum exopolysaccharide production.

Different sugar components *viz*: sucrose, dextrose, mannitol *etc*. as a sole source of carbon[32,33] yields EPS than cell biomass[34]. Different carbon sources supplemented at 1% level promoted the bacterial growth and EPS production by a *Rhizobium* sp. to different extent and mannitol was the most effective promoter[31]. Utilization of different carbon sources for the growth and EPS production by *Rhizobium* sp. was reported[35]. Higher concentrations of polysaccharides were obtained when carbon: nitrogen ratio was higher in growth medium[35].

Nitrate, glycine and ammonium conjugate were most preferred as nitrogen source whereas sulphate of nitrogen is less preferred by most of the *Rhizobium sp*[35]. Exopolysaccharide production is favored under conditions of nitrogen limitation but excess sugars remaining in the growth medium can be used specifically for polysaccharide synthesis[9]. However, thiamine hydrochloride and nicotinic acid increased EPS production by different species of *Rhizobium*[16]. Exopolysaccharide production also varies with time, as a function of growth phase, for many bacteria. For many rhizobacterial species, growth and exopolysaccharide production occur simultaneously, because EPS biosynthesis being growth-associated[35]. Several studies have indicated that the EPS yields vary with bacterial growth phase, while EPS composition remains constant through the batch cycle of growth[35].

The present study was aimed towards determining the effect of different carbon, nitrogen and vitamine sources on exopolysaccharide production of *Rhizobium* species isolated from root nodule of redgram (*Cajanus cajan*) in *in vitro* culture.

Materials and Methods

Microorganism, Medium and Growth Condition

The symbionts were isolated from fresh, healthy, surface sterilized and pink coloured root nodules of redgram (*Cajanus cajan*) and was grown in axenic culture. The medium selected for the growth of bacteria was yeast extract mineral medium of Skerman with 1% mannitol and having 0.1% $CaCl_2.H_2O$ instead of $CaCO_3$ and NaCl at neutral pH (7.0). The isolates were grown in 100 mL conical flasks containing 20 mL medium with three replicates of each at 30±2°C on a rotary shaker for 30 h. the growth of the strains were measured turbidometrically by a colorimeter at 540 nm.

Isolation of Exopolysaccharide

The cell free culture filtrate after centrifugation at 10000 × g for 20 min was used for extracting EPS. For precipitating the polysaccharides the 3 volumes of acetone were added to a unit volume of cell free culture filtrate. After centrifugation at 6000 × g for 10 min, the precipitated polysaccharides were collected and suspended in (1 mL) distilled water. Three volume of acetone was added to the dissolved polysaccharide for reprecipitation and centrifuged; the process was repeated thrice. After that EPS solution in distilled water was used for taking reading.

Estimation of exopolysaccharide by spectrophotometry

The dissolved polysaccharide solution was used for estimation of EPS by phenol sulphuric acid method[36]. Reaction mixture (1:1) in a test tube contained 1 mL of EPS solution and 1 mL of aqueous phenol. The 5 mL of H_2SO_4 was added to it. The tubes were allowed to stand for 20 min after vigorous shaking. The absorbency was measured at 490 nm. The EPS solution in distilled water was used as control and determined against glucose standard.

Optimization of Culture

To check maximum growth and EPS production by strains, different carbon, nitrogen and vitamin sources

were used. Individual sources were added separately to the tryptophan supplemented basal medium. The effect on growth and EPS production were recorded.

Statistical analysis

The data was analyzed statistically of as per the procedure prescribed for complete randomized design by using the software Statistical Package for the Social Sciences (SPSS).

Result and Discussion

Effect of carbon sources on growth and EPS production

The symbionts were isolated from fresh and healthy mature nodules of redgram. The medium used for pure culture was yeast mannitol agar congored (YMAC) medium. All the three isolated symbionts were identified after submission of 16s rRNA sequence to NCBI as *Rhizobium sps.* strain CHRS-7, RAB-1 and RAN-1with gene bank (NCBI) accession number as MH636329, MH636773 and MH541051 respectively**.** The growth (OD) and corresponding EPS production was found that all the strains could reach their stationary phase of growth at 36 h (Fig. 1) of incubation, while maximum EPS production by CHRS-7, RAB-1 and RAN-1 were 58 µg/mL, 53 µg/mL and 70 µg/mL respectively, occurred after 48 h of incubation and thereafter EPS production decreased (Fig. 2). The increase in EPS production after attending stationary phase of growth was reported earlier[37]. The growth and EPS production started simultaneously that indicates some parts of EPS might be acting as primary metabolite and helps in nodulation.

Production of EPS, specific productivity by the isolates and their growth was studied with the replacement of mannitol by eight different carbon sources (1%) along with mannitol (1%) from the Yeast Extract Mineral medium of Skerman, Maximum Growth (1.59), EPS (75 µg/mL) of CHRS-7 was recorded in glucose, RAB-1 had maximum growth (1.39), EPS (71 µg/mL) and specific productivity (51.08) was obtained in manitol and RAN-1 had maximum growth (1.74), EPS (85 µg/mL) and specific productivity (48.85) was obtained in sucrose (Table 1).

The most preferred carbon sources for each strain (manitol, glucose, and sucrose) were tested with a gradual increase (0.5 to 2.5%) along with a control set with no carbon source. The EPS production of CHRS-7 was maximum (89 µg/mL) in glucose (1.5 %), whereas growth was maximum in glucose 1% (Fig. 3), RAB-1 produced maximum EPS (78 µg/mL) and growth (1.47) at 2% manitol (Fig. 4) and RAN-1 produced maxium EPS (85 µg/mL) and growth (1.99) in 1.5% and 1% respectively, sucrose (Fig. 5). The *Rhizobium* sp. metabolized different carbon sources for the production of EPS were also reported [22].

Effect of nitrogen source on growth and EPS production

The six different nitrogen sources (0.1%) were used to record the effect on EPS production and the growth (Table 2). The growth of strain CHRS-7 was highest (5.30) with glycine (0.1%) followed by other nitrogen sources: KNO_3>L-aspergine>$NaNO_3$> $(NH_4)_2SO_4$> NH_4Cl, respectively, and least growth was recorded in control (1.27). Growth of CHRS-7 with each of the six sources of nitrogen was significantly different than others. Exopolysacharide production of CHRS-7 was estimated highest (76 µg/mL) in glycine followed by other nitrogen sources in the order KNO_3> NH_4Cl > $NaNO_3$> L-aspergine=$(NH_4)_2SO_4$ respectively. Lowest (34 µg/mL) was recorded in control. The production of EPS with glycine was significantly higher than other nitrogen sources whereas EPS production in $NaNO_3$, KNO_3, > NH_4Cl was statistically at par. Specific productivity was highest in NH_4Cl and control. The growth of strain RAB-1 was maximum (4.20) with $NaNO_3$ (0.1%) whereas lowest (1.19) was recorded in control. The suitability of N sources for the growth

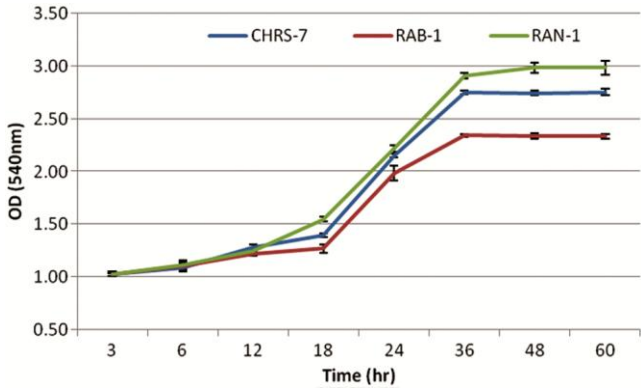

Fig. 1 — Growth curve of strains at different time interval

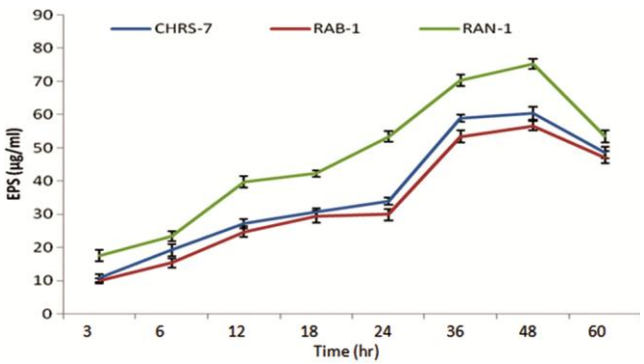

Fig. 2 — Production of EPS by strains at different time interval

Table 1 — Effect of different carbon sources on growth and EPS production by *Rhizobium* sp. (strain CHRS-7, RAB-1 and RAN-1) in culture

	CHRS-7			RAB-1			RAN-1		
Carbon Source (1%)	OD (540 nm)	EPS (µg/mL)	Specific Productivity	OD (540 nm)	EPS (µg/mL)	Specific Productivity	OD (540 nm)	EPS (µg/mL)	Specific Productivity
Control	1.13 ^{g} (±0.01)*	19^{e} (±1.76)	17	1.09^{c} (±0.01)	14^{ef} (±1.2)	13	1.19^{f} (±0.01)	24^{f} (±1.53)	20
Glucose	1.58 ^{a} (±0.02)	75^{a} (±1.53)	47	1.19^{b} (±0.02)	69^{a} (±1.2)	58	1.72^{ab} (±0.01)	58^{b} (±1.53)	34
Galactose	1.37 ^{c} (±0.01)	62^{b} (±1.53)	45	1.18^{b} (±0.01)	57^{b} (±1.2)	49	1.65^{d} (±0.02)	48^{c} (±1.45)	29
Fructose	1.45 ^{b} (±0.01)	50^{c} (±1.45)	35	1.20^{b} (±0.01)	29^{d} (±1.76)	24	1.69^{bc} (±0.01)	56^{b} (±1.20)	33
Maltose	1.13 ^{g} (±0.01)	21^{e} (±1.20)	19	1.11^{c} (±0.01)	16^{e} (±1.15)	14	1.27^{e} (±0.01)	34^{d} (±1.20)	27
Lactose	1.14 ^{fg} (±0.02)	23^{e} (±1.73)	20	1.12^{c} (±0.01)	14^{ef} (±1.20)	13	1.29^{e} (±0.02)	29^{e} (±0.88)	22
Sucrose	1.17 ^{f} (±0.01)	20^{e} (±1.76)	18	1.19^{b} (±0.01)	12^{f} (±0.88)	10	1.74^{a} (±0.02)	85^{a} (±0.58)	49
Manitol	1.27 e (±0.01)	34^{d} (±2.08)	27	1.39^{a} (±0.01)	71^{a} (±1.2)	51	1.66^{cd} (±0.01)	51^{c} (±1.76)	31
Starch	1.33 d (±0.02)	47^{c} (±2.03)	35	1.21^{b} (±0.01)	37^{c} (±1.0)	31	1.65^{d} (±0.01)	49^{c} (±1.53)	30
LSD (*P* =0.05)	0.04	5.04	–	0.03	3.6	–	0.03	4.0	–

*Data in the parenthesis represents the standard error. All the values for growth OD and EPS production sharing different alphabet in superscript indicate significant differences following multiple comparisons (Duncan's multiple range test) for the carbon sources

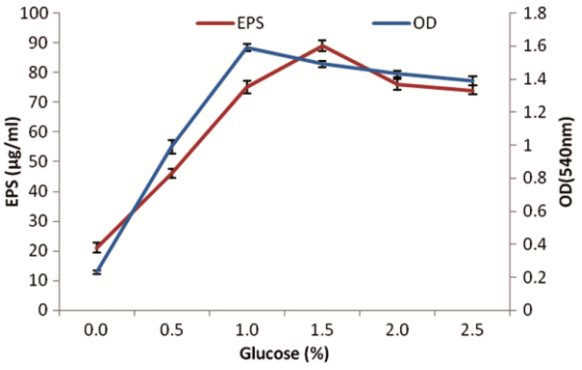

Fig. 3 — Growth and production of EPS of CHRS-7 in different concentrations of glucose

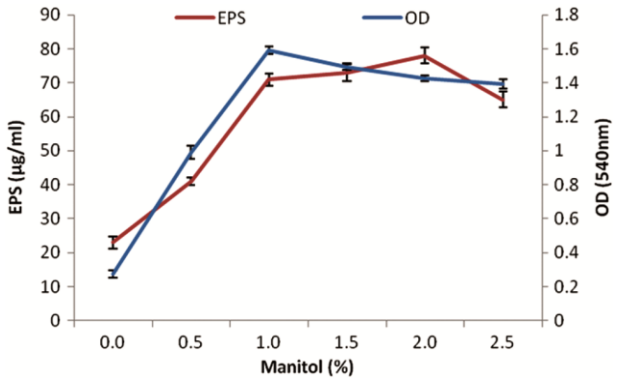

Fig. 4 — Growth and production of EPS of RAB-1 in different concentrations of manitol

was found to be in the order: $NaNO_3$> L-aspergine> $NaNO_3$> Glycine> NH_4Cl>$(NH_4)_2SO_4$, respectively. The EPS production was also highest (71 µg/mL) in KNO_3 (0.1%) followed by L-aspergine> Glycine> NH_4Cl> KNO_3>$(NH_4)_2$ SO_4, respectively and lowest (29 µg/mL) was observed in control. The specific productivity was highest (24) in $(NH_4)_2$ SO_4 (0.1%), NH_4Cl and control the lowest (16) was calculated with L-aspergine and KNO_3 treated medium. Similarly, among the N-sources RAN-1 also grew well in 0.1% KNO_3 with OD (4.29) and EPS production was highest (123 µg/mL). Lowest OD (3.54) and EPS (89 µg/mL) was recorded in 0.1% $(NH_4)_2$ SO_4. In non nitrogenous media, the OD (2.76) and EPS (51 µg/mL) production were lowest. The specific productivity was also highest (29) with KNO_3. The most preferred nitrogen source of CHRS-7, RAB-1 and RAN-1 were glycine, $NaNO_3$ and KNO_3, respectively (Figs. 6-8).

The preferable sources were also tested with graded doses (0.1, 0.2, 0.3, 0.4 and 0.5%) and the control was without any nitrogen source. Both CHRS-7 and RAN-1 produced maximum EPS (µg/mL) (108 and 119) and OD (5.41 and 4.21) at 0.2% of glycine and KNO_3, respectively. The EPS production decreased with increased in glycine concentration. Kumar and Ram[38] reported that the EPS produced in the presence of glycine by different *Rhizobium* sp. which supported the results of the present study. The strain RAB-1 produced maximum EPS (97 µg/mL) and OD (4.14) at 0.3% $NaNO_3$. The *Rhizobium* sp. utilizes different nitrogenous compounds for their growth was reported by Nirmala *et al*[39].

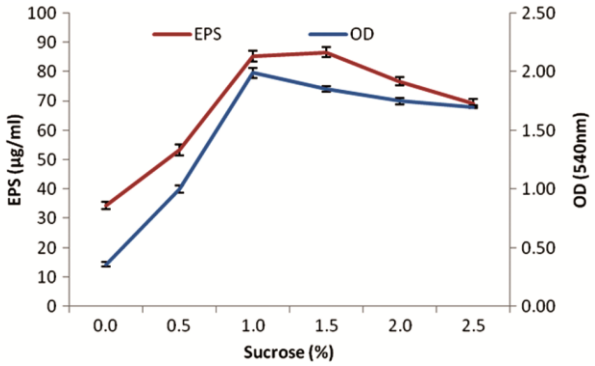

Fig. 5 — Growth and production of EPS of RAN-1in different concentrations of sucrose

Effect of vitamine source on growth and EPS production

The effect of five different vitamins (1 µg/mL) on growth and EPS production of the three isolates *vis*. CHRS-7, RAB-1 and RAN-1 were monitored. When the medium was supplemented with biotin (1 µg/mL) the maximum EPS (107, 99 and 130 µg/mL), OD (1.40, 1.49 and 1.47) and specific productivity (77, 67 and 89) were recorded in CHRS-7, RAB-1 and RAN-1, respectively (Table 3). The growth of CHSR-7 was lowest (1.26) in the medium added with nicotinic acid and EPS production was also lowest (40 µg/mL) in control. The specific productivity was highest (77) in biotin. The growth of RAB-1 was lowest (1.32) in riboflavin supplemented medium, whereas EPS (36 µg/mL) production and specific productivity (47)

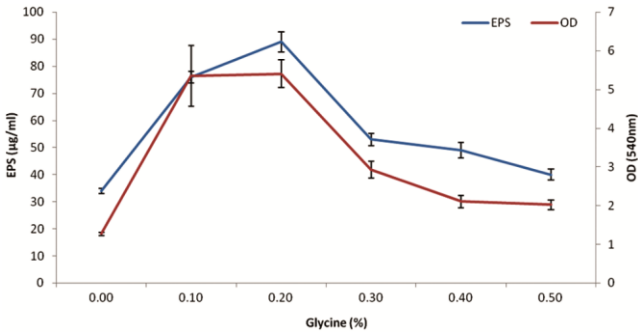

Fig. 6 — Growth and production of EPS of CHRS-7 in different concentrations of glycine

Table 2 — Effect of different Nitrogen sources on growth and EPS production by *Rhizobium* spp. (strain CHRS-7, RAB-1 and RAN-1) in culture

	CHRS-7			RAB-1			RAN-1		
N-Source (0.1%)	OD (540 nm)	EPS (µg/mL)	Specific Productivity	OD (540 nm)	EPS (µg/mL)	Specific Productivity	OD (540 nm)	EPS (µg/mL)	Specific Productivity
Control	1.27^{g} (±0.01)*	34^{d} (±1.24)	27	1.19^{g} (±0.02)	29^{e} (±0.74)	24	2.76^{f} (±0.02)	51^{e} (±0.86)	18
KNO_3	4.52^{b} (±0.03)	69^{b} (±1.86)	15	3.71^{c} (±0.02)	58^{cd} (±1.67)	16	4.29^{a} (±0.02)	123^{a} (±2.11)	29
$NaNO_3$	3.90^{d} (±0.05)	65^{b} (±1.57)	17	4.20^{a} (±0.03)	71^{a} (±1.88)	17	3.90^{d} (±0.03)	107^{b} (±1.46)	28
L-aspergine	4.15^{c} (±0.02)	54^{c} (±1.33)	13	4.05^{b} (±0.06)	65^{b} (±2.72)	16	4.20^{b} (±0.03)	110^{ab} (±2.04)	26
Glycine	5.30^{a} (±0.04)	76^{a} (±1.76)	14	3.50^{d} (±0.03)	62^{bc} (±1.92)	18	4.11^{c} (±0.04)	99^{c} (±2.29)	24
$(NH_4)_2 SO_4$	2.17^{f} (±0.02)	54^{c} (±1.88)	25	2.27^{f} (±0.03)	55^{d} (±1.39)	24	3.29^{g} (±0.02)	96^{c} (±1.17)	27
NH_4Cl	2.50^{e} (±0.15)	68^{b} (±2.88)	27	2.52^{e} (±0.03)	61^{bc} (±1.35)	24	3.54^{e} (±0.03)	89^{d} (±1.53)	27
LSD (P =0.05)	0.19	5.63	–	0.10	5.34	–	0.08	5.18	–

*Data in the parenthesis represents the standard error. All the values for growth OD and EPS production sharing different alphabet in superscript indicate significant differences following multiple comparisons (Duncan's multiple range test) for the Nitrogen sources

Table 3 — Effect of different vitamin sources on growth and EPS production by *Rhizobium* sp. (strain CHRS-7, RAB-1 and RAN-1) in culture

	CHRS-7			RAB-1			RAN-1		
Vitamine (µg/mL)	OD (540 nm)	EPS (µg/mL)	Specific Productivity	OD (540 nm)	EPS (µg/mL)	Specific Productivity	OD (540 nm)	EPS (µg/mL)	Specific Productivity
Control	1.37^{a} (±0.03)*	40^{e} (±1.31)	29	1.39^{bc} (±0.03)	36^{e} (±0.95)	26	1.29^{c} (±0.03)	60^{d} (±1.39)	47
Nicotinic acid	1.26^{b} (±0.02)	72^{c} (±1.66)	57	1.41^{ab} (±0.02)	67^{d} (±2.32)	47	$1.34b^{c}$ (±0.02)	118^{b} (±2.24)	88
Thiamine	1.39^{a} (±0.02)	72^{c} (±2.52)	52	1.43^{ab} (±0.03)	86^{b} (±1.92)	60	1.43^{ab} (±0.04)	107^{c} (±1.46)	75
Riboflavin	1.32^{ab} (±0.03)	63^{d} (±2.63)	47	1.32^{c} (±0.02)	76^{c} (±1.47)	58	1.41^{ab} (±0.03)	110^{c} (±2.04)	78
L-ascorbic acid	1.33^{ab} (±0.03)	86^{b} (±1.76)	64	1.45^{ab} (±0.02)	82^{b} (±1.72)	57	1.43^{ab} (±0.02)	103^{c} (±2.81)	72
Biotin	1.40^{a} (±0.04)	107^{a} (±2.36)	77	1.49^{a} (±0.03)	99^{a} (±1.47)	67	1.47^{a} (±0.03)	130^{a} (±2.85)	89
LSD (P =0.05)	0.09	6.46	–	0.07	5.23	–	0.09	6.90	–

*Data in the parenthesis represents the standard error. All the values for growth OD and EPS production sharing different alphabet in superscript indicate significant differences following multiple comparisons (Duncan's multiple range test) for the Vitamin sources

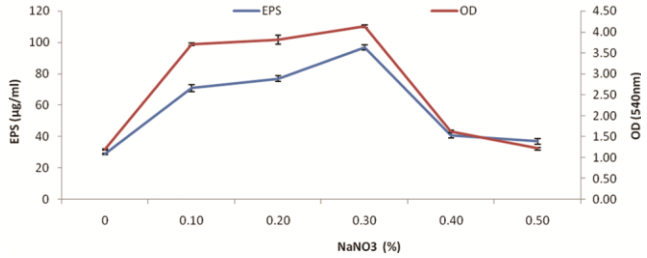

Fig. 7 — Growth and production of EPS of RAB-1 in different concentrations of $NaNO_3$

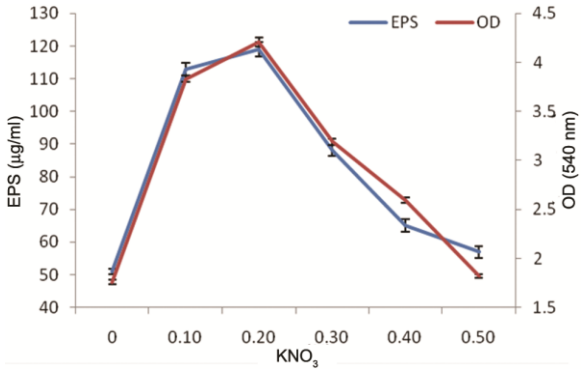

Fig. 8 — Growth and production of EPS of RAN-1 in different concentrations of KNO_3

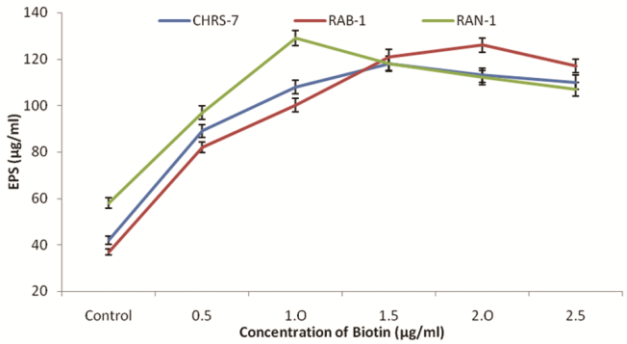

Fig. 9 —EPS production of strains in different concentrations of biotin

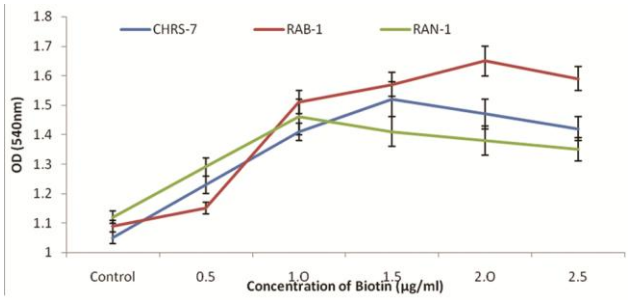

Fig. 10 — Growth of strains in different concentrations of biotin

was lowest in control. Isolate RAN-1 recorded lowest growth (1.29), EPS (60 µg/mL) production and specific productivity in control.

The preferred vitamin (biotin) was tested at various concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 µg/mL. The EPS productions by all the three strains were different at different concentrations of biotin. The highest EPS production (118, 126 and 129 µg/mL) was attended by CHRS-7, RAN-1 and RAB-1 in 1.5, 2.0 and 1.0 µg/mL biotin, respectively (Fig. 9). Lowest was observed in control. A similar trend was also observed with OD (Fig. 10). The EPS by *Rhizobium* sp. (VMA301) in biotin (1.5 µg/mL) was reported[14].

Conclusion

All the three rhizobia strains isolated from root nodule of redgram could produce copious amount of exopolysaccharides in *in vitro* culture using different carbon, nitrogen and vitamin sources. The glucose, manitol and sucrose were the preferable carbon sources and glycine, sodium nitrate and potassium nitrate preferable nitrogen sources for CHRS-7, RAB-1 and RAN-1, respectively. All the strains preferred biotin as vitamin source for producing exopolysacharide.

Acknowledgment

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