Extracellular polysaccharide production by *Rhizobium sp*. with their plant growth promoting activity isolated from *Cajanus cajan* root nodules

[1] Bhairav Prasad, *[2]Mohit Mishra [3]Ashish Saraf & [4]Kamini Kashyap

[1]Vidya Jyoti Institute of Higher Education, SAS

Nagar -140508 (Punjab) India

[2]Amity University Chhattisgarh, Amity Institue of Biotechnology, Raipur 493225

[3]MATS University Pagariya Complex Pandri Raipur

[4]SUS College of Research and Technology, SAS Nagar (Punjab), India

Corresponding author: mishra.msbiotech@gmail.com

1.0 ABSTRACT

Rhizobium is soil bacteria which are characterized morphologically as gram negative, small rods and motile. During nodule formation EPS is secreted by *Rhizobium* in addition to many plant growth promoting factor to access the root hair infection. A total 14 isolates were recovered and were designated as RZK1- RZK14. These isolates were morphologically and biochemically characterized and were gram negative, rod. Then EPS was isolated and estimated from these all 14 isolates and the highest producer viz. RZK7, RZK9 and RZK13 were subjected for further studies. The amount of EPS produced by these isolates was 500, 512 and 515µg/ml respectively. RZK13 grown in YEMM containing 2% mannitol have produced the highest amount of EPS (645µg/ml). PGPA tests were also performed for these three selected isolates. RZK7, RZK9 and RZK13 were found to be positive for siderophore test, phosphate solubilization, ammonia production, HCN production, Zn solubilization and cell wall degrading enzymes. Bioinoculation of isolate RZK13 was done in soyabean seeds. Plants grown from inoculated seeds shown fast growth in comparison to the control seeds. In 20 days, plants originated from the inoculated seeds have attained height of 18cm whereas control plants have attained height of 12cm only.

Key words: Exopolysaccharides, PGPA, Rhizobium

2.0 Introduction

Exopolysaccharides are high-molecular-weight polymers that are composed of sugar residues and are secreted by a variety of microorganism including bacteria, fungi and yeast into the surrounding environment. Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS). Exopolysaccharides generally consist of monosaccharides and some non-carbohydrate substituents such as acetate, pyruvate, succinate, and phosphate. Owing to the wide diversity in composition, exopolysaccharides have found multifarious applications in various food and pharmaceutical industries. Many microbial EPS provide properties that are almost identical to the gums currently in use. With innovative approaches, efforts are underway to supersede the traditionally used plant and algal gums by their microbial counterparts. Moreover, considerable progress has been made in discovering and developing new microbial EPS that possess novel industrial significance (Suresh and Mody, 2009).

The sensory benefits of the exopolysaccharides produced by rhizobacteria are well established and essential for colonization of rhizosphere and roots for the infection of plant and provide the microbiota for nitrogen-fixing bacteria to plant roots and soil particles (Ullrich, 2009). Rhizobia are Gram-negative bacteria that can exist either as free-living bacteria or as nitrogen-fixing symbionts inside root nodules of leguminous plants. The composition of the rhizobial outer surface, containing a variety of polysaccharides, plays a significant role in the adaptation of these bacteria in both habitats. Among rhizobial polymers, exopolysaccharide (EPS) is indispensable for the invasion of a great majority of host plants which form indeterminate-type nodules. Various functions are described to this heteropolymer, including protection against environmental stress and host defense, attachment to abiotic and biotic surfaces, and in signaling. The synthesis of EPS in rhizobia is a multi-step process regulated by several proteins at both transcriptional and post-transcriptional levels. Also, some environmental factors viz. carbon source, nitrogen and phosphate starvation, flavonoids and stress conditions viz. osmolarity, ionic strength affect EPS production (Janczarek, 2011). In nitrogen-fixing bacteria which establish symbiosis with legumes forming indeterminate-type nodules, EPS is additionally indispensable for successful infection of host plant roots (Skorupska *et al.*, 2006). Rhizobial EPS is also very important for proper biofilm formation both on abiotic surfaces and on host plant roots, being the major component of the biofilm matrix (Downie, 2010; Rinaudi and Giordano, 2010). The exoploysaccharides production by *Rhizobium sp*. is a very complex and multistep process which are regulated at gene level and various proteins are involved. The principal aim of the present investigation are the isolation of some potential exoploysaccharides producing *Rhizobium* bacterial isolates that should mitigate the requirement of plant growth promoting activity and the use of potential isolates as bioinoculant.

3.0 Materials and methods

The present work was carried out in the Microbiology laboratory of Shaheed Udham Singh College of Research and Technology, Tangori, Mohali, Punjab (India).

3.1Materials used

3.2 Chemicals and glassware:

All the chemicals used for preparing reagents and solutions were procured from Hi- media, sd-fine chemicals and of AR grade. For isolation and characterization, dehydrated media used were procured from Hi-media and were used such as per the manufacturer's. All the glasswares used like test tubes, beakers, conical flasks, petripaltes, etc. were of borosilicate glass.

3.4Methodology

Collection of samples

A total of 14 leguminous plants were collected from Himachal Pradesh and Punjab which were used for isolation of *Rhizobium sp.* These leguminous plants were uprooted carefully so that their roots can be prevented from physical damage from the fields and transferred into the plastic bags and transported immediately to the laboratory for further processing.

3.5 Selective Isolation of *Rhizobium sp.* from leguminous plants

Uprooted leguminous plants were brought to the laboratory. Roots were washed properly to remove soil attached to the roots. Healthy, pink unbroken nodules were selected and washed with water properly. Nodules were then immersed in 3% H_2O_2 for about 5 minutes for surface sterilization. Then repeated washing of nodules was done with distilled water to get rid of sterilizing agent. Then root nodules were crushed with the help of sterilized glass rod in 1ml D.W. to make uniform suspension, its serial dilutions were made upto 10^{-5} and 0.1ml of root nodule suspension was spread over YEMA plates and incubated at 26-30°C for 3 to 5 days.

3.6 Maintenance and Purification of isolates

Morphologically distinct colonies were observed and identified and then were picked with help of sterilized inoculating loop and streaked over YEMA plates to obtain pure culture. The purified isolates were then streaked on nutrient agar slants and stored at 4°C in a refrigerator. The revival of the culture was done on monthly basis to maintain the purity.

3.7 Morphological characterization

Morphological characterization of isolates was done according to the methods by Aneja, (2003) by observing the colony morphology such as shape, colour, margin, elevation, and surface. The cell morphology like shape, arrangement was studied by gram staining and carbol fuchsin staining.

3.8 Biochemical Characterizations

All the biochemical tests were performed according to the method described by Aneja (2003). Different biochemical tests were performed for biochemical characterization of the isolates. Biochemical tests which were performed were IMViC, Catalase, Nitrate reductase, Urease, Carbohydrate fermentation

test, Citrate utilization, Motility test, Skim milk test, Hydrogen sulfide production test, Casein hydrolysis etc.

3.9 Confirmatory test for the *Rhizobium sp.*

YEMA was enriched with bromothymol blue (BTB) indicator for differentiating between fast growers and slow growing *Rhizobium* sp. All isolates were streaked on YEMA medium contain BTB indicator and incubated at 26-30°C. Fast growers were visualized after 2-3 days and produced acidic reaction due to which colour of medium changed to yellow. Whereas slow growers take much longer time than fast growers and produce alkaline reaction due to which medium turns blue.

3.10Measurement of bacterial growth

YEMM media was prepared for all the total 14 isolates. In 100ml of flask 25 ml of YEMM was poured and autoclaved. After autoclaving each isolates were inoculated and flasks were marked properly. Then these flakes were placed in the rotary shaker at 120 rpm for 72hrs. at 26-30°C. After 72 hrs growth of each isolates were measured at 610nm.

3.11Exo-polysaccharides isolation

10ml of the 72 hr bacterial growth was taken in the centrifugation tubes and was centrifuged at 10000X rpm for 20 min to obtained cell free filtrate, which was used for extracting EPS. 1ml of the filtrate was taken and 3 ml of acetone was added for precipitating the polysaccharides and further centrifuged at 6000 X rpm for 10min. The precipitated was re- dissolved in 1ml D.W for washing. This step was repeated two to three times. After washing the precipitate was again dissolved in D.W. and used for the estimation of EPS.

3.12 Estimation of EPS

Estimation of EPS was done by phenol-sulfuric acid method described by Dubois *et al.* (1965). 1ml of EPS sample obtained in the above step was taken in centrifuge tube. To this 1ml of phenol and 5ml of concentrated H_2SO_4 was added. Shaken vigorously and allowed to stand for 20 min. Absorbance was taken at 540nm against EPS in D.W. as control. Amount of EPS was determined against glucose standard curve.

3.13 Effect of Different Carbon sources on EPS production

EPS production was done by formulating medium with different carbon sources. The different carbon sources taken were lactose, glucose, mannitol and sucrose. Potential producers (RZK7, RZK9 and RZK13) of EPS selected at the above step were selected and grown in different carbon sources. YEMM media was supplemented with different carbon sources (2%w/v). Three flasks were control, contain no carbon source. These were autoclaved, inoculated with loopfull of cultures and incubated in rotary shaker at 26-30°C for 72hrs. After incubation, EPS produced was isolate and estimated according to the method explained in section 3.7 and 3.8. Potential isolate producing highest amount EPS was selected for the plant

growth promoting activities.

3.14 Plant Growth Promoting Activity

The following plant growth promoting tests were performed:

3.15 Siderophore production

Siderophore production test was assayed on chromo zaurole 'S' agar. The selected isolates were spot inoculated in CAS agar medium and incubated aerobically at 28±2°C for 4-5 days. Production of yellow-orange halo zone around the growth considered positive test for siderophore production. Their zone of diameter was recorded.

3.16 Phosphate solubilization

The selected isolates were spot inoculated in Pikovskaya agar medium and incubated aerobically at 26-30°C for 3 to 5 days. Production of clear zone around the growth considered positive for phosphate solubilization. Their zone of diameter was recorded.

3.17 Hydrogen cyanide production

The selected isolates were streaked on King's B agar medium. A Whatmann's filter paper was immersed in 0.5% picric acid and placed on the lid on the petriplate and incubated aerobically at 26-30°C for 3 to 5 days. Change in colour Whatmann filter paper was considered positive for HCN production.

3.18 Ammonia production

Peptone water was prepared by dissolving 1gm of peptone in 100ml of D.W. It was autoclaved and inoculated with selected isolates. Incubated at 26-30°C for 3-4 days and after incubation 0.5ml of Nessler's reagent was added to each test tube. Production brown or yellow colour indicated positive test for ammonia production.

3.19 Zinc solubulization test

In 100ml of NA, 0.1% of Zn was supplemented and autoclaved. Sterilized media was poured into petriplates and allowed to solidify. After solidification media containing Zn was spot inoculated and incubated aerobically at 26-30°C for 3 to 5 days. Clear zone or any halo zone appeared around colony which indicated positive test.

3.20 Cell wall degradation enzymes

Protease activity was determined by the formation of clear zone in skimmed milk agar. Skimmed milk agar was prepared, autoclaved and poured in the petriplates. After solidification of the media spot inoculation was done and incubated at 26-30°C for 3 to 5 days. Clear zone appeared around the colonies indicated positive test.

Cellulose degrading activity was checked by preparing CMC agar plates and spot inoculating the isolates. Incubation done at 26-30°C for 3 to 5 days. Clear zone formed around colonies after incubation indicated a positive test.

3.21 Bio-inoculation of Soybean seeds with *Rhizobium sp.* (RZK13)

Potential producer of EPS and plant growth promoting activities isolate RZK13 was selected and grown on YEMA plates and incubates at 26-30°C for 3 to 5 days. Using scalpel, scrapped the growth on the plates and suspended in D.W., 50g of sugar was taken and added in 500ml of water. Sugar solution was heated for 15 min and then 200gm of gum Arabic was added into hot sugar solution. This solution was allowed to cool at room temperature. Inoculum (bacterial growth) which was prepared was mixed in the solution. Then soybean seeds were added in the solution and mixed the seeds with this solution properly with the help of hands wearing gloves, so that slurry should be uniformly coated over seeds. These seeds were transferred to thick sheets and then sown in the pot. Another pot was taken which contained unincolated seeds and growth was observed after 4 days interval.

4.0Results and Discussion

Isolation of *Rhizobium sp.*

A total of 14 isolates were obtained from 14 leguminous plants on YEMA plates. Colonies obtained on the YEMA plates were colorless, have gummy like secretions around the colonies, which were purified further, by streaking on the YEMA plates. These purified cultures were subcultured on the YEMA slants and preserved and maintained at below 4°C in the refrigerator. Similarly, Nirmala *et al.,* (2011) isolated *Rhizobiium* strains from root nodules of *Vigna mungo* and were designated as *Rhizobium* Vm1 to Vm10. The isolated strains were identified as species of *Rhizobium* having colourless, gummy like secretions around the colony.

4.1 Characterization of Isolates

4.1.1. Morphological characterization

For the characterization of isolates, shape, elevation, surface and colour of colonies are observed. For the morphological characterization of isolates carbol fuchsin and gram staining were performed. All the isolates recovered from the sites were having smaller, medium and large, gummy colonies, circular, and regular in shape and were gram negative. Similarly, Sharma *et al.,* 2010, configuration of the *Rhizobium* colonies grown on YEMA medium was circular as well as regular. The margin of all the isolates was entire except the isolate G, which was wavy. The elevation was convex, raised and umbonate. The colour of the colonies varied from translucent to opaque. Ogutcu *et al.* (2009) isolated 17 isolates from wild chickpea. All were found to have circular colonies, with regular borders, flat in elevation, creamy colour, showing high production of mucus production.

4.1.2.Biochemical characterization

Different biochemical tests were performed for confirming that the isolates. All the isolates were found to be positive for catalase, H_2S production, motility test, carbohydrate fermentation, nitrate reduction and urease, while negative for indole, MRVP, Citrate, and gelatin hydrolysis. According to Ghachande and Khansole (2011) the bacterium showed positive test for citrate, production of ammonia and catalase activity. Nitrate is reduced to nitrite producing ammonia.

Neal and Walker (1935) suggested rapid nitrate utilization by slow growing root nodule bacteria. Mahana *et al.* (2000) reported catalase activity in some isolates from Vigna mungo. The bacterium is negative for MR-VP and indole reaction. Similarly, Grahm and Parker (1964) did not observe MR reduction in all the isolates of seven *Rhizobia* groups. Similarly Shahzad *et al*. (2012) out of fifty (50) samples of root nodules from alfalfa (*medico sativa*), twenty five 25 were found positive for the presence of *Sinorhizobium meliloti*, after screening through a series of various biochemical and Sugar fermentative tests. The samples were found negative for Methyl Red (MR), Voges-Proskauer (VP), Indole, Citrate utilization test, and Gel liquefaction tests.

4.1.3 Growth of Isolates on Bromothymol Blue (BTB)

Based on observations after incubation YEMA at 28±2˚C medium that is enriched with BTB. It was concluded that the isolates of *Rhizobium sp.* which could grow within 2-3 days of incubation, turned colour of medium from green to yellow. Colour changed due to production of acid and are known as fast growers, whereas isolates of *Rhizobium sp* which took longer time for growth i.e. 6-8 days and turned colour of medium from green to blue are slow growers and this change in colour of medium was due to alkaline reaction. Similarly, Ogutcu *et al.*(2009) reported that after 3-5 days of growth YMA at 28°C all strains isolated by him acidified the medium which is indicated by bromthymol blue and colony diameter ranged from 2-5mm in Bergey's Manual.

Similarly, Shahzad *et al.* (2012) reported that he positive samples were streaked on Bromothymol blue (BTB) added Yeast Extract Mannitol (YEM) selective media for further confirmation, showed moist and gummy colonies on BTB after incubation for 48 hrs at 28°C and surrounding medium plate were yellow due to acid production by the *Sinorhizobium meliloti*.

Table 4.4 Growth of Isolates on BTB

Isolates	Growth of *Rhizobium sp*. on BTB	
	Fast /Slow growers	YEMA turned yellow/ Blue
RZK1	S	B
RZK2	F	Y
RZK3	F	Y
RZK4	F	Y
RZK5	S	B

RZK6	F	Y
RZK7	F	Y
RZK8	F	Y
RZK9	F	Y
RZK10	F	Y
RZK11	S	B
RZK12	F	Y
RZK13	F	Y
RZK14	F	Y
***S**: Slow growers (take 6 to 8 days for growth); **F: Fast growers (take 2-3 days for growth)		***B**: Blue (medium colour turned blue); *Y: Yellow (medium turned yellow)

4.1.4 Measurement of bacterial growth and Estimation of EPS

Growth of *Rhizobium sp*., that we got as isolates from different leguminous plants were grown on flasks in YEMM and after 72hrs, this was used for measuring the growth of isolates by checking at 610nm. The EPS estimation was done by following the procedure and taking absorbance at 540nm. The amount of EPS produced was extrapolated with glucose standard curve. On the basis of absorbance taken at 540nm, highest values of EPS production have been seen in RZK7, RZK9 and RZK13 with optical density 1.991, 2.020 and 2.047 respectively with EPS yield of 500, 512 and 515µg/ml. The growth yield and the EPS production by the various isolates is depicted in table 4.5. These three potential producers of EPS were chosen for carrying out optimization with different carbon sources. Similarly, Nirmala *et al.,* (2011) reported that the among 10 *Rhizobium* strains tested, the *Rhizobium* Vm6 produced maximum amount of 1680µg/ml EPS on Yeast Extract mineral medium. EPS productions in more than twenty Rhizobium strains have studied by van Workum and Kijne, (1998). Bhaskar (2003) isolated 150 bacterial isolates which were obtained from various natural samples and departmental culture collections were screened for EPS production. The morphology and the gram characteristics of some of the isolates and their EPS production estimated after 48 h of incubation in 1% glucose supplemented BSS medium. According Mukherjee *et al.*, (2011) *Rhizobium sp*. has been known to produce large amount of EPS that might be involved in symbiosis of inoculant with host plant. The EPS is an extracellular polysaccharides produced by the large number of rhizobium sp. associated with the leguminous plant, which might be helping in the nodule formation and provide protection from the external environment.

Table 4.5 Growth profile and EPS measurement

Isolates of Rhizobium spp.	Growth measurement (610 nm)	EPS (540nm)	EPS Production µg/ml
RZK1	0.781	0.960	255
RZK2	0.862	0.967	259
RZK3	0.819	0.949	249
RZK4	0.871	0.975	263
RZK5	0.673	0.964	254
RZK6	0.807	0.966	258
RZK7	**1.877**	**1.991**	**500**
RZK8	0.819	0.977	264
RZK9	**1.801**	**2.020**	**512**
RZK10	0.855	0.979	265
RZK11	1.898	1.960	495
RZK12	1.853	1.313	454
RZK13	**1.854**	**2.047**	**515**
RZK14	0.884	1.21	405

4.1.5 Effect of different carbon sources on EPS production

RZK7, RZK9 and RZK13 were grown on different carbon sources which included glucose, lactose, sucrose, mannitol and one set remained as control containing no carbon source and incubated at 28±2°C. After incubation in rotary shaker at 120 rpm with an incubation time of 72hrs. the bacterial growth was checked by taken absorbance at 610 nm and the EPS production was estimated. The Results obtained are depicted in figures shown below.

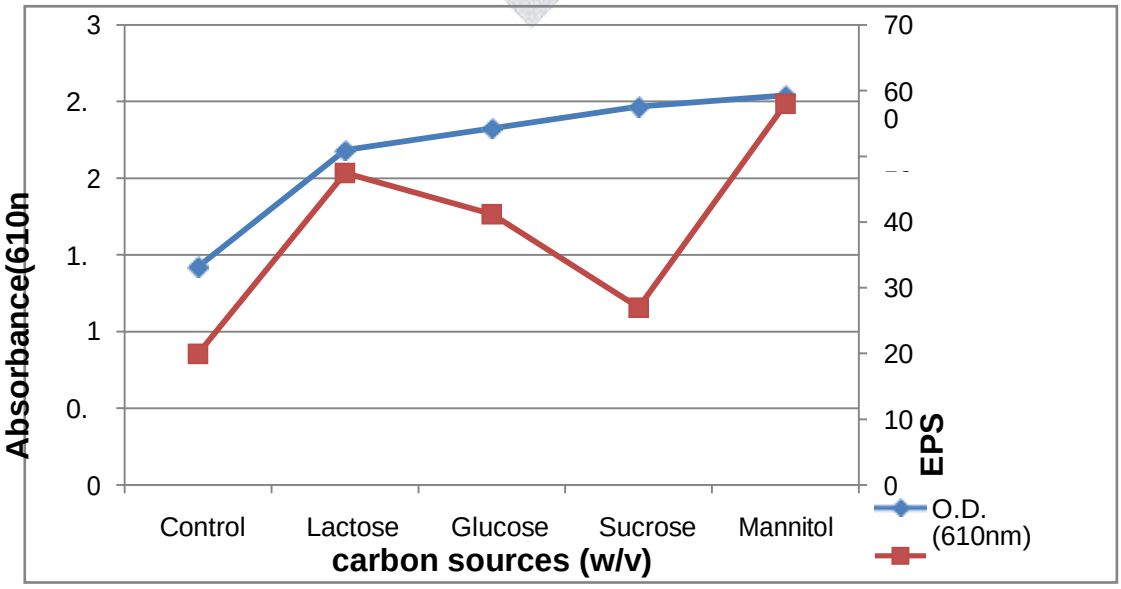


Fig4.1: EPS produced by RZK7

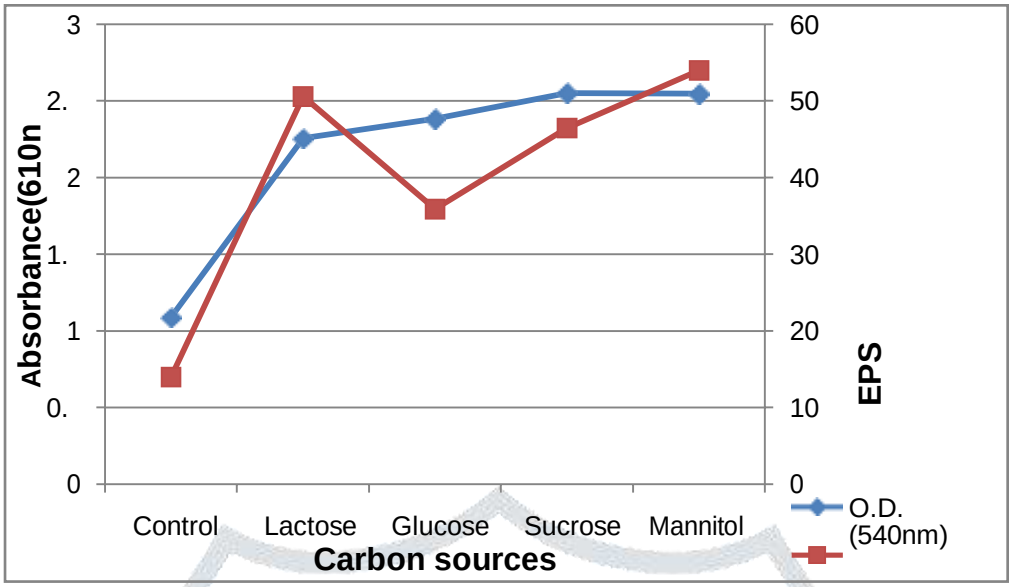


Fig4.2: EPS produced by RZK9

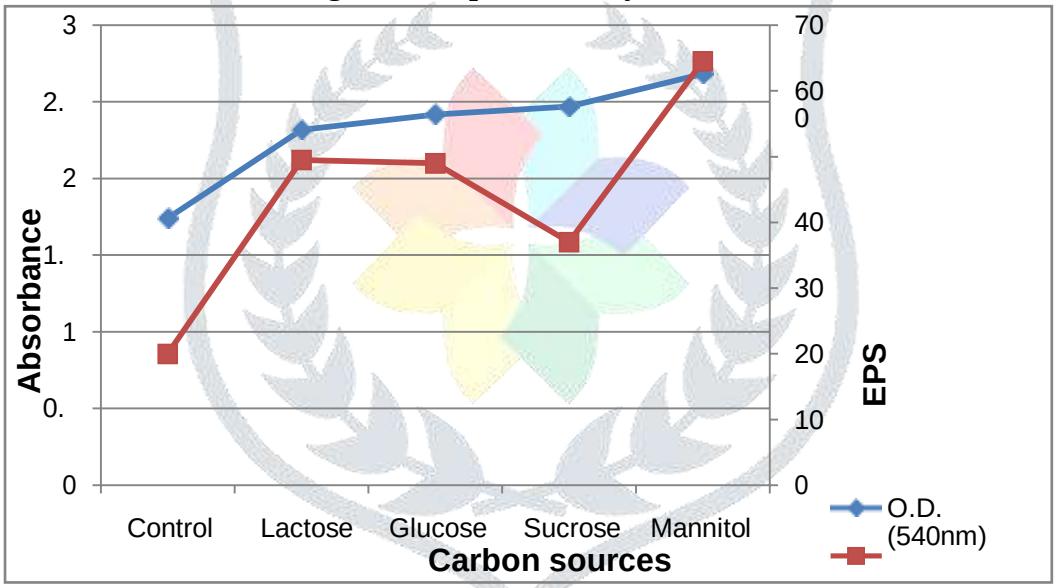


Fig4.3: EPS produced by RZK13

Different carbon sources were taken for finding out the best carbon source for increase in the production of EPS. It was observed that 2% of carbon sources taken enhanced the production of EPS and bacterial growth. Among different carbon sources, 2% mannitol was the most suitable promoter for EPS production in RZK7, RZK9 and RZK13 with amount of 580, 540 and 645µg/ml respectively. Similarly, Nirmala *et al*., (2011) reported that among the eight tested carbon sources (1%) enhanced both bacterial growth and EPS production to different extents. Optimum concentration of mannitol required for EPS production was found to be 2 %. Bhaskar (2003) reported that *Marinobacter sp*. showed maximum growth when the medium was supplemented with 3% glucose. However, EPS concentration was maximal when the culture was grown in 1% glucose. Thereafter, EPS concentrations did not increase with the increase in glucose concentrations, although cell growth increased upto 3% glucose. After observing the figure 4.1, 4.2 and 4.3 it can be

concluded that RZK13 grown in YEMM containing 2% mannitol have produced the highest amount of EPS. Moreover, on the basis of observations obtained it can be concluded that mannitol is best carbon source for the growth of *Rhizobium* sp., because RZK7 RZK9 and RZK13 have shown maximum growth on 2% mannitol.

4.1.6 Plant growth promoting activity

Different PGPA tests were performed to know about the plant growth promoting activities of isolates these tests were performed to know about some substances which may be released by *Rhizobium sp.* and promote the growth of plants.

Siderophore production

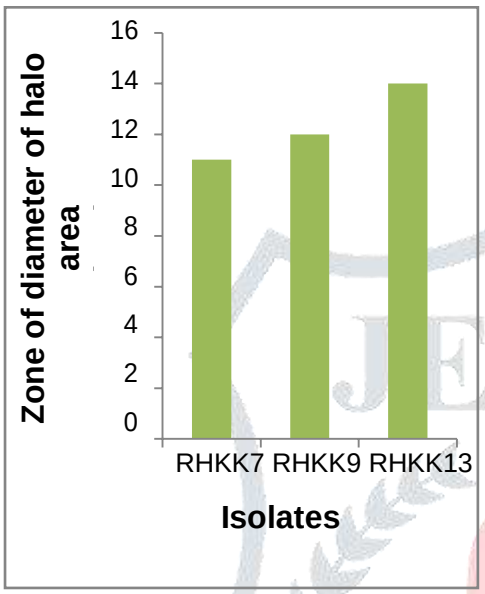
In siderophore test, yellow colored halo zone was formed around the growth in RZK7, RZK9 and RZK13 on CAS agar medium. Therefore, the three isolates have shown positive test for siderophore production. The frequency of siderophore production among the isolates were ranged from 11-14mm. Size of halo zone for RZK7, RZK9 and RZK13 was 11mm, 12mm and 14mm respectively. Siderophore is a type of biocontrol mechanism mechanism which is produced by plant growth promoting rhizobacteria. The result is depicted in the fig.4.4. Similar results were also obtained by Joseph *et al.*, (2007) while screening the bacteria from the root nodules and found positive test for siderophore production. Siderophore are the compound that chelates iron and other metals and it can suppress diseases by supplying limited number essential trace elements in natural habitats and it also synthesize antimicrobial compounds. It can also act as host resistance. Chaicharn *et al.*, (2008) also suggested that siderophore producers have been reported well for plant growth promotion especially in neutral to alkaline soil. They screened about 220 phospahte solubilizing bacteria and found that they have good prospect to improve plant growth, especially in the soil with large amount of precipitation. Iron is limiting bioactive metal in the soil and essential for the growth of soil microorganisms. Nagrajkumar *et al.*, (2004) also reported that rhizobacteria can acquire iron by the production of siderophores, through which they could compete for iron with soil borne pathogen.

4.1.7 Phosphate solubilization

The three isolates selected for the PGPA tests have shown positive test for phosphate solubilization. Clear zones appeared around the colonies produced on pikovskaya's agar. The range of phosphate solubilizing activities of all the three isolates viz, RZK7, RZK9 and RZK13 was 12, 10 and 18mm respectively. The result is depicted in fig 4.5.

According to Vessey *et al.*, (2003) appearance of halo zones around some of the colonies suggested phosphate solubiling ability. Greater the halo zone diameter strongest the phosphate solubilizing activity of bacteria. It has been reported that higher concentrations of phosphate solubilizing bacteria are commonly found in rhizospheric soil and non rhizospheric soil. Islam *et al.*, (2007) reported that the solubilization of Phosphorous by bacteria like *Rhizobium* or any other sp present in the rhizosphere shows that it helps in increasing the nutrient availability to host plants. According to Halder *et al.*, (1991), *Rhizobium* is able to

solubilize phosphate. The role of phosphate solubilization in plant growth promotion often overshadowing other plant beneficial activities expressed by phosphate solubilization bacteria. Simialrly, Kumari *et al.,* (2010) all the five isolates of *Indigofera* species were also tested for solubilization of tri-calcium phosphate on Pikovskaya's medium using spot inoculation method. After 5–7 days of incubation the Pikovskaya's medium plates were observed for the formation of clear zone of phosphate solubilization around the colonies.



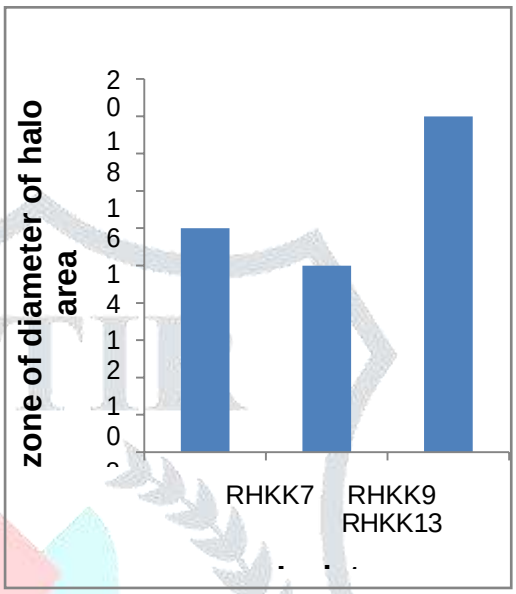


Fig 4.4 Siderophore production **Fig 4.5 P-solubilizing activity of isolates.**

As, from the above results, it is clear that RZK13 shows highest halo zone as compared to RZK7 and RZK9. So, it can be concluded that RZK13 have active mode of phosphorus solubilization and siderophore production.

4.8 HCN production

RZK7, RZK9 and RZK13 were grown on King's B and found that all the three isolates were positive test for HCN production (Slide 4.14). Kremer *et al.,* (2006), reported that, isolates show positive reaction by developing varying amount of colour intensity developed over pictrates or Na_2CO_3 impregnated paper. On the basis of their result HCN production greater than 5 nmoles/mg cellular proteins may contribute to growth inhibition of seedlings. Albert and Bob (1987) isolated 98 bacteria from rhizosphere of wheat and potato on TSA. The main reason of HCN production is its antagonistic property.

Ammonia production

RZK7, RZK9 and RZK13 were inoculated into peptone water. After incubation, Nessler's reagent was added and colour of broth changed from yellow to dark yellow or brown, which indicated that these three selected isolates of *Rhizobium* were positive for ammonia production test (Slide 4.15). According to Joseph *et al*., (2007) ammonia production was detected in 95% of isolates of *Bacillus* followed by *Pseudomonas*

(94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45.0%). The rizospheric bacteria having capacity to convert the elemental nitrogen into the ammonia. Ammonia is very good fertilizer for the plants that can be directly involved in the amino acid synthesis.

4.9 Zn solubilization

The three selected isolates i.e. RZK7, RZK9 and RZK13 were grown on NA supplemented with 0.1% Zn. Growth of the three isolates was observed on the agar supplemented with Zn with clear zone around the colonies. Halo zones formed around the colonies suggested positive test. All the isolates were found to be positive for Zn solubilization. Woo *et al.,* (2010) suggested that zinc is a macro element present in the soil, and helps in the plant growth such cell division and other metabolic activity. Wilting and chlorosis are two impacts of absence of this trace element.

4.10 Cell wall degradation test

Protease activity was performed by inoculating RZK7, RZK9 and RZK13on skim milk agar and incubated for appearance of clear zone around colonies. Clear zone formed around the colonies. Similarly, for checking cellulose activity, RZK7, RZK9 and RZK13 were grown on CMC agar and incubated for the appearance of clear zone. RZK7, RZK9 and RZK13 could produce halo zones on CMC agar. So, RZK7, RZK9 and RZK13 were positive for cellulose activity. These cell wall degrading enzyme play important role in the establishment of symbiotic relationship between the microorganisms and higher plant. These enzymes also facilitates the absorption of many minerals from the soil that directly enhance the growth and productivity of plant, it also helps in the interaction of nutrients to plant. So many degrading enzymes has inhibitory activity against the pathogenic organisms. Similarly, Farah *et al.,* (2006) reported that cell wall degrading enzymes help in establishment of symbiosis between microorganism and plant and enhance the growth of plant. He depicted these results during screening of bacteria from the rhizospheric soil which were positive for cellulose and protease activity.

4.11 Bioinoculation of Rhizobium (RZK13) into soyabean

Soyabean seeds were inoculated with the potential isolate i.e. RZK13 which was selected after optimization and these seeds were sown in the pot. And another pot was sown with the soyabean seeds which were not inoculated with *Rhizobium.* Proper watering was done and growth was observed and shown in table given below.

Table 4.6 Growth Pattern of Soyabean

Days	Height of bioinoculated plants (cm)	Height of Control plant (cm)
4th day	Seedlings germinated	Seedlings germinated (poor)
8th day	5.0	2.0
12th day	8.0	5.5
16th day	12	7.0
20th day	17	11

As result shown in above table 4.8, it can be concluded that soyabean inoculated with RZK13 isolate of *Rhizobium sp.* has shown positive results because inoculated seeds have shown fast growth as compared to the control plant. Therefore, RZK13 is found to be having great potential for the plant growth promoting activities. As it produced the maximum amount of EPS, as well as also showing positive test for the plant growth promoting activities viz. siderphore production, phosphate solubilization, Zn solubilization, HCN production and cell wall degrading enzymes.

Slide 4.16 BioInoculated seeds

Slide 4.17 Control Plant

Acoording to Roomi *et al.,* (1994), *Rhizobium* inoculums appear to be most suitable method of inducing root nodule formation due to which better growth of plants was observed. Choure and Agarwal (2011) reported that *Rhizobium* strains isolated from their native field are successful biofertilizers which increase the crop yield. Behl *et al.,* (2007) reported that microorganisms which are used as bioinoculants for wheat are free living, phosphate solubilizing and nitrogen fixing microorganisms. According to Sethi and Adhikary (2012) *Rhizobium* and *Azotobacter* has been used for improvement of crop productivity by

inoculation process, which has been reported extensively successful. There is large scale production of biofertilizer using these microorganisms which are available at commercial level for field application.

5.0 CONCLUSION

Rhizobium is a soil bacteria which are characterized morphologically as gram negative, small rods and motile. There are different factors of *Rhizobium* which help in the nodulation process, EPS is one of the factor and other factors are nod gene, K-antigen and lipopolysaccharides and cyclic glucans. Extracellular polysaccharides are high molecular weight polymers that are composed of sugar residues and are secreted by microorganisms in their environment. EPS provide functional and structural stability to the bacteria. During nodule formation EPS is secreted by *Rhizobium* to access the root hair infection. It is also reported that the *Rhizobium* produce an arsenal of plant growth promoting factors. Bioinoculation of isolate RZK13 was done in soybean seeds. Plants grown from inoculated seeds shown fast growth in comparison to the control seeds. In 20 days, plants originated from the inoculated seeds have attained height of 18cm whereas control plants have attained height of 12cm. From the above study it can be concluded that the root nodules of leguminous plants are rich in rizobium bacteria and a potential sources for these bacteria. The isolates of rhizobium produced EPS and potentially having the plant growth promoting activities. The isolates RZK13, produced higest amount of EPS, 645µg/ml with the YEMM medium supplemented with 2%(w/v) mannitol, along with incubation period of 72 hrs, at 30±2 ^{O}C. Therefore with these properties the isolate RZK13 can be exploiting as potential bioferilizer. Experimentally *Rhizobium* has been proved a good biofertilizer which have no ill effects on soil or on the environment. It increases the productivity without any adverse effect. On the other hand chemical fertilizers have many harmful effects over environment. Chemicals fertilizers when used in excessive can make change in pH, texture of soil which can be undesirable, and can also produce harmful health effects like allergies, asthma, skin problems to human. But biofertilizer have no harmful effects over environment. *Rhizobium* as biofertilizer can make abundant nutrients available to plants; it fixes nitrogen into the soil. So, these help in increasing the fertility of the soil. But biofertilizer like *Rhizobium* fixes nitrogen in the soil directly from the atmosphere, and deals with plant and soil only, no bad effects are produced to humans or environment. EPS produced by *Rhizobium sp* can be used in food industries and in gel making.

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