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Screening and Characterization of Exopolysaccharide Substance Producing Bacteria and Its Role in Enhancement of Plant Growth

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Abstract

Microbes play important role in plant growth as it provides various substances((PGPR) required for plant to grow. Many organisms are responsible for that function depends on nature plant and organism. In present study exopolysaccharide producing organism were screened and checked how it contribute for plant growth. Exopolysaccharide substance producing microbes were isolated from soil sample of various region of valsad and vapi region. Total 14 exopolysaccharide producing microbes were screened positive for PGPR activity. These organisms were checked for various PGPR activity such as indole acetic acid production, ammonia production, zinc solubilization, siderophore production, HCN production, antagonistic test. Pot experiment was performed in which length of root and shoot were measured. Bacterial isolate I-25 gave maximum PGPR activity hence proved to be maximum contributor for plant growth which was proved by pot experiment. So, this organism was proved to be novel for plant growth.

Keywords

Bacteria, PGPR, Exopolysaccharide, pot experiment.

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1. INTRODUCTION:

Exopolysaccharides are formed from monosaccharide residues of sugar and sugar derivatives secreted by bacteria in external environment which provides nutrition to plants and protect it from various conditions, prevent leakage etc. [7,10]. Plant growth promoting rhizobacteria (PGPR) of many plant species and confer beneficial effects, such as increased plant growth and reduced susceptibility to diseases caused by plant pathogenic microbes as well as pathogenic eukaryotes [14]. Formation, structure, and stability of bioggregates with an emphasis on the composition and distribution of extracellular polymeric substances (EPS) and their role in bioaggregation. EPS are composed of a mixture of macromolecules including proteins, polysaccharides, humic like substances, and nucleic acids, which entrap the microbial cells in a three-dimensional matrix [9]. The rhizobia inside the nodules then convert nitrogen into ammonia for uptake by host plants, while legumes provide nutrients to rhizobia [1]. The wide scale application of PGPR may decrease the global dependence on agricultural chemicals. Furthermore, it is a technology which is readily accessible to farmers in both developed and developing countries [3].

2. MATERIALS AND METHODS

2.1 Sample Collection

Total of 14 soil samples were collected from various rice field Soil, wheat field Soil, pulses field Soil, garden soil, and agricultural farms soil of Valsad, Vapi, Gujarat, India. were used to isolate the mucoid colony producing bacteria.

2.2 Isolation of Exopolysaccharide producing microorganisms:

Isolation of exopolysaccharide producing microorganisms were done using nutrient agar plate (Peptone 10.0gm, Beef extract 3.0gm, Sodium chloride 5.0gm, Agar powder 30gm, Dissolved in 1000 mL of Distilled water, pH 7.0) [14].

2.3 Screening and Characterization of Exopolysaccharide producing microorganisms:

Characterization was done by selecting mucoid (exopolysaccharide producing) colony and capsule staining by Maneval's staining method (Maneval staining method).

2.4 Estimation of Exopolysaccharidse substance:

2.4.1 Preparation for inoculum

Inoculum was prepared in a nutrient broth, containing 50 mL of nutrient broth in 100 mL conical flask. Take a loopful culture from preserved culture plate and inoculate it into nutrient medium incubated for 24 hrs in shaking incubator at 30°C, 150 rpm for 24 hrs. The freshly grown culture with 1.0 O.D. at 600 nm was used as inoculum for the estimation of exopolysaccharide substance [12].

2.4.2 Precipitation of EPS

Exopolysaccharide in the nutrient medium was precipitated using chilled ethanol.

First step: 10 mL sample was added to centrifuge tube and centrifuged at 5,000 rpm for 20 minutes. Then supernatant was transfered in another tube and pellets were discarded.

Second step: Equal amount of chilled ethanol was added and incubated in refrigerator at 4°C for 30 min, the precipitated EPS was centrifuged for 20 minutes and the supernatants were discarded.

Third step: The pellet obtained was dried in filter paper in oven at 60 - 70°C and dry weight of exopolysaccharides was taken to estimate the yield of EPS in medium [12].

2.5 Plant Growth Promoting Activity of Exopolysaccharide Producing Microorganisms:

2.5.1 Analysis of Phosphate Solubilizing Activity:

The qualitative as well as quantitative analyses of phosphate solubilizing activity of the selected isolates were conducted by plate screening method and broth culture method.

2.5.1.1 Qualitative assay of phosphate solubilizing efficiency and phosphate solubilizing activity by screened microorganisms:

All screened organisms were tested for phosphate solubilization on pikovskaya's medium. Screened organisms were inoculated on pikovaskaya agar plate (Yeast Extract 0.05, Dextrose 1.0, Tri Calcium Phosphate 0.5, Ammonium Sulphate 0.05, Potassium Chloride 0.02, Magnesium Sulphate 0.01, Manganese Sulphate 0.0001, Ferrous Sulphate 0.0001, Distilled Water 1000 mL, Agar powder 30.0gm pH 7.0 ± 0.2) and incubated at 37˚C for 3-4 days. After incubation zone of phosphate solubilization was measured [16].

2.5.1.2 Quantitative assay of phosphate solubilizing efficiency and phosphate solubilizing activity of screened microorganisms:

Quantitative estimation of Phosphate solubilization for previously screened organisms in qualitative phosphate solubilization assay was carried out. The Erlenmeyer flask (250 mL) containing inoculated into 100 mL pikovskaya's PVK medium (Yeast Extract 0.05, Dextrose 1.0, Tri Calcium Phosphate 0.5, Ammonium Sulphate 0.05, Potassium Chloride 0.02, Magnesium Sulphate 0.01, Manganese Sulphate 0.0001, Ferrous Sulphate 0.0001, Distilled Water 1000 mL, pH 7.0 ± 0.2) with 0.5 % (w/v) Tri-calcium phosphate was sterilized at 121^{0}C after 15 min at 15 psi. Medium was inoculated with 1 mL of culture (Optical density-1.0), incubated at 37^{0}C. At different time intervals 1,3,5,7, and 10 days the culture broth samples were drawn and phosphate solubilization was estimated [16].

Phosphorous Assay: After incubation, flask was harvested, and sample was centrifuged at 10,000 rpm for 15 minutes.1mL of the supernatant was taken and mixed with 1mL of ammonium molybdate-vanadate and 3mL of distilled water. Uninoculated broth served as control. The absorbance was taken at 470nm and compared with standard curve of KH_2PO_4 by reduced Vanadate-molybdate (VM) method [6].

2.5.2 Zinc solubilization assay

All the isolates were inoculated on to modified pikovskaya's PVK Agar medium (Yeast Extract 0.05, Dextrose 1.0, Tri Calcium Phosphate 0.5, Ammonium Sulphate 0.05, Potassium Chloride 0.02, Magnesium Sulphate 0.01, Manganese Sulphate 0.0001, Ferrous Sulphate 0.0001, Distilled Water 1000 mL, pH 7.0 ± 0.2) containing 1% insoluble zinc compound . All the plates were incubated for 48 hours at 30^{0}C. Zone of solubilization around the colony was measured and considered as zinc solubilizing bacteria [8].

2.5.3 Indole Acetic Acid (IAA) Production:

2.5.3.1 Qualitative Analysis of Indole Acetic Acid (IAA) production:

Bacterial cultures were grown in nutrient broth amended with L-tryptophan (amended with 5 mM L-tryptophan, 0.065 % sodium dodecyl sulphate and 1% glycerol) for 24 hrs at 30^0C under shaking conditions. Followed by aaddition of 2 drops of o-phosphoric acid and 4mL of salkowski reagent (50mL,35% per choric acid, 1mL of 0.5M $FeCl_3$) incubated at room temperature for 20 minutes (in dark) for the development of pink colour. Absorbance was taken at 535nm.Concentration of indole acetic acid was estimated by preparing calibration curve using Indole Acetic Acid as standard (10-100µg/ml) [2].

2.5.3.2 Quantitative Analysis of Indole Acetic Acid (IAA) production:

Bacterial cultures were grown in nutrient broth amended with L-tryptophan (amended with 5 mM L-tryptophan, 0.065 % sodium dodecyl sulphate and 1% glycerol) for 72hrs at 37ºC under shake conditions. After incubation cultures were centrifuged at 3000rpm for 30 minutes.

The supernatant (2mL) was mixed with two drops of o-phosphoric acid and 4mL of salkowski reagent (50mL,35% per choric acid, 1mL of 0.5M $FeCl_3$) incubated at room temperature for 20 minutes (in dark) for the development of pink colour. Absorbance was taken at 535nm.Concentration of indole acetic acid was estimated by preparing calibration curve using indole acetic acid (IAA, Hi-media) as standard (10-100µg/ml) [2].

2.5.4 Siderophore production

All exopolysaccharide producing microorganisms were screened for siderophore production using CAS medium.

2.5.4.1 Qualitative Estimation of Siderophore Production:

Preparation of Chrome azurol S (CAS) reagent:

2mM of CAS solution was mixed with 1mM $FeCl_3$ solution and 0.04% HDTMA (hexodecyltrimethyl ammonium bromide). Reagent turns blue in color.

Chrome azurol S (CAS) Assay:

Screened organisms were inoculated in CAS agar plate incubated at 30^0C for 3-5 days. Development of yellow to orange color indicates siderophore production [2].

2.5.4.2 Quantitative Estimation of Siderophore Production (Chrome-azurol-S(CAS) liquid assay method):

0.1 mL of cell free extract of culture supernatant was mixed with 0.5 mL Chrome-azurol-S(CAS) assay solution. 10 µl of shuttle solution (0.2M 5-Sulfosalicyclic acid) was added. It was kept at room temperature for 10 minutes and absorbance was recorded at 630 nm. The minimal medium was used as a blank and the reference(r) was prepared using exactly the same components except the cell free extract of culture supernatant. [13]

2.5.5 Nitrogen fixation

Bacterial isolates were cultured on Nitrogen free by Jensen's medium (Sucrose 20gm, Dipotassium phosphate 1gm, Magnesium sulphate 0.5gm, Sodium chloride 0.5gm, Ferrous sulphate 0.1gm, Sodium molybdate 0.005gm, Calcium carbonate 2.0gm, Agar powder 15gm, Distilled water 1000mL). Incubated for 2 or 3 days at 30^0C. Growth of microorganism on nitrogen lacking Jensen medium indicates nitrogen fixing ability of microorganism [8].

2.5.6 Ammonia production

Bacterial isolates were inoculated in peptone broth (peptone water). 1% inoculum was added to 5mL of peptone water in each tube. Broth was incubated for 24 hours in test tubes at 30°C. 0.5mLNessler's reagent was added in each tube. Absorbance was measured at 660nm. Development of brown–yellow color indicates ammonia production [2].

2.5.7 Hydrogen Cyanide Production

Bacteria were inoculated on nutrient agar plate containing 4.4gm/L of glycine. Filter paper strips soaked in picric acid reagent were placed in the lid of each Petri dish. Plates were incubated at 30˚C for 5-7 days. Development of yellow to light brown color indicates HCN production [16].

2.5.8 Antagonistic Test

Antagonistic effect of test organism against fungi *Aspergillus niger.*, *Fusarium* sp., and *Penicillium* sp. was examine on Czapek Dox Agar plate (sucrose 3.0gm, $NaNO_3$ 2.0gm, K_2HPO_4 1gm, $MgSO_4.7H_2O$ 0.5gm, KCl 0.5gm, $FeSO_4$ 0.01gm, Agar Powder 30gm, Distilled Water 1000mL, pH 7.0). The plates were incubated at 30°Cfor 5-7 days. Antagonism was measured by measuring zone of inhibition [11].

2.6 Pot experiment

A bioassay-based determination of the plant growth promotion ability of the isolates was conducted using *Vigna radiata* (mung bean) seedlings in sterile soil.

The mung bean seeds were surface sterilized in 70% ethanol for 2 minutes and in 0.2% $HgCl_2$ for 5 minutes followed by 10 times washing with sterile distilled water.

Pure cultures were grown in nutrient broth at 28°C. The surface sterilized seeds were coated by immersing in the appropriate PGPR suspension for 45 minutes at room temperature, air-dried, and sown for germination activity in plastic pot containing soil. Control seeds were treated with sterile distilled water. 10 seeds per pot was remain constant for

whole experiment. The soil was moistened to 50% of its water-holding capacity.

After 15 days' Morphological characteristics such as plant height, root length of each plant was recorded. At harvest, the root system was separated from shoots, and both were oven dried overnight at 65°C and dry weights were recorded against the control [2].

3. RESULTS AND DISCUSSION

3.1 Isolation of Exopolysaccharide producing microorganisms:

Total 26 bacteria were isolated from various rhizospheric soil samples of Valsad and Vapi region.

3.2 Screening and characterization of Exopolysaccharide producing microorganism:

From 26 bacterial isolate, 14 were able to secrete exopolysaccharide substance and all of them were gram negative rod shape organisms which was also characterized by presence of capsules.

Table 1: Exopolysaccharide production by screened microorganisms

Sr.No.	Bacterial Isolate	EPS yield(mg/mL)		
		24 hours	48 hours	76 hours
1	I-1	0.022	0.046	0.041
2	I-3	0.022	0.031	0.03
3	I-5	0.011	0.134	0.117
4	I-8	0.008	0.012	0.067
5	I-11	0.009	0.108	0.312
6	I-12	0.014	0.054	0.384
7	I-13	0.006	0.027	0.296
8	I-15	0.001	0.017	0.187
9	I-19	0.01	0.032	0.388
10	I-20	0.01	0.002	0.264
11	I-22	0.03	0.069	0.265
12	I-24	0.001	0.558	0.101
13	I-25	0.027	0.002	0.243
14	I-26	0.019	0.558	0.033

3.3 Plant growth promoting Activity of Exopolysaccharide substance producing microorganism:

3.3.1: Phosphate Solubilization:

3.3.1.1: Qualitative Analysis of Phosphate solubilization:

From 14 screened organisms, 7 bacteria were able to solubilize phosphorous in which I-25 gave maximum zone of solubilization up to 0.6cm which enhance plant growth by proving phosphorous required for its growth.

3.3.1.2: Quantitative Analysis of Phosphorous Solubilization:

From above 7 bacteria which gave positive results for phosphorous solubilization, isolate I-25 gave maximum phosphorous solubilization up to 0.251µg/ml after 24 hours.

Figure 1: Qualitative Analysis of Phosphorous Solubilization by I-25

Figure 2: Quantitative Analysis of Phosphorous Solubilization I-25

3.3.2 Zinc solubilization:

From 14 screened bacteria, 4 isolate which gave positive results for I-25 gave maximum was able to solubilise zinc.

Figure 3: Zinc solubilization by isolate I-25.

3.3.3 Indole Acetic Acid (IAA) Production:

3.3.3.1: Qualitative Analysis of Indole Acetic Acid (IAA) Production:

From, 14 screened organisms all were able to produce indole acetic acid (IAA).

Fig 4: Quantitative Analysis of Indole Acetic Acid (IAA) Production

3.3.3.2: Quantitative Analysis of Indole Acetic Acid (IAA) Production:

From above 14 bacteria which gave positive results for indole acetic acid (IAA) production, maximum indole acetic acid production was given by Isolate-3, Isolate-11, Isolate-26.

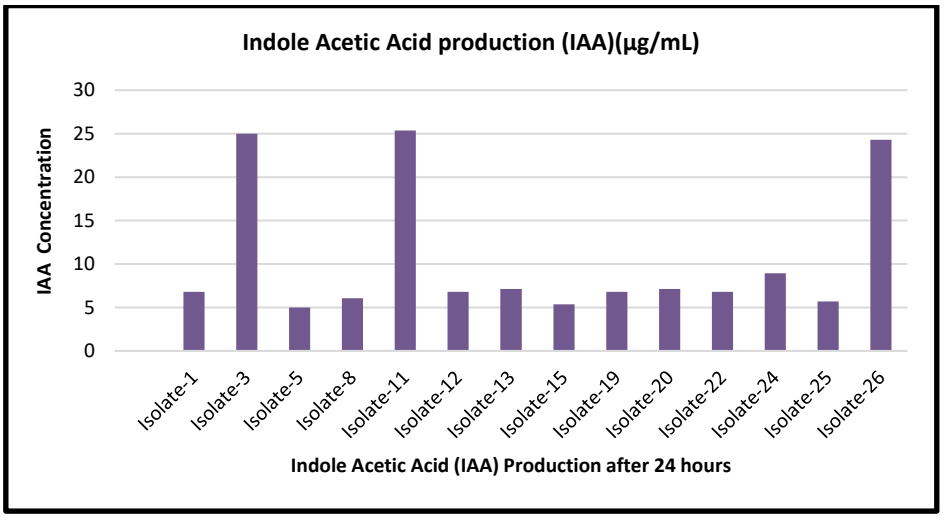


Figure 5: Quantitative analysis of Indole Acetic acid production

3.3.4 Ammonia production:
From 14screened organisms 12 bacteria were able to produce ammonia.

3.3.5 Nitrogen fixation:
From 14 screened organisms, 13 were able to fix nitrogen.

3.3.6 Hydrogen Cyanide (HCN) Production:
From 14 screened organisms only isolate I-12 were gave hydrogen cyanide (HCN) production which helps in enhancement of plant growth.

3.3.7 Siderophore Production:
From 14 screened bacterial isolate, only 2 bacterial isolate I-1 & I-25 were able to produce siderophore which provides binding of ion to plant and enhance its growth.

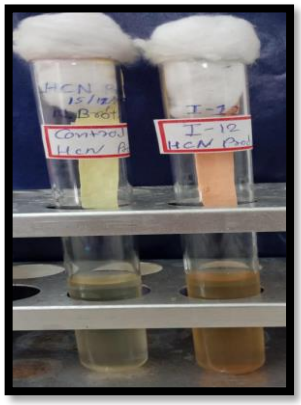
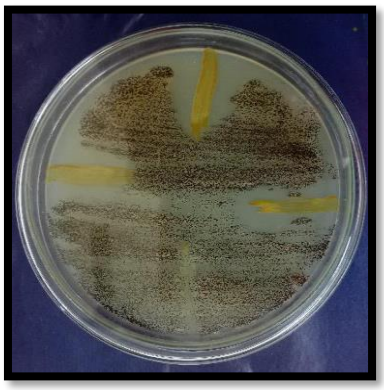


Figure 3.6: HCN Production by bacterial isolate I-12

Figure 3.7: Siderosphore Production

3.3.8 Antagonism assay against Phyto- pathogenic fungus:
Antagonistic effect against fungi *Fusarium* sp., *Aspergillus Niger,* was observed by isolate I-25. And antagonistic effect against *Penicillium* sp. was not observed by any of 14 screened organisms.



Test Fungi: *Aspergillus niger*

Test Fungi: *Fusarium spp.*

Figure 3.8: Antagonistic effect of isolate I-25

3.4: Pot Experiment:
On treatment of seeds in bacterial culture before sown helps in enhancement of plant growth was proved by increase in length as compare to control.

Table 2: Root and shoot length of plant after treating with specific organism

Bacterial Isolate	Root Length	Shoot Length
Control	1.2	13.1
I-1	2.4	14.2
I-3	3.2	15.2
I-5	1.3	10.3
I-8	2.1	17.1

I-11	4.2	16.3
I-12	2.1	13.5
I-13	4.3	14.3
I-15	5.1	15.1
I-19	4.2	15.6
I-20	3.1	11.1
I-22	5.1	15.4
I-24	3.2	16.2
I-25	6.1	18.4
I-26	2.3	14.1

4. CONCLUSION

Extra polymeric substance (EPS) secreted by bacteria are having many applications in field of agriculture, medicine, environment pollution control etc. it helps bacteria to adhesion to the surface and for colonization. Studies show that bacterial isolate I-25 were able to produce Indole Acetic acid and able to solubilize phosphorous and zinc in higher amount which is required for plant growth. I-25 were able to produce siderophore which provides iron to plants and increase its binding capacity. This isolate can able to produce ammonia and able to fix nitrogen from environment which help plant to gain nutrient required for its growth. It shown antagonistic effect against fungi *Fusarium* sp. and *Aspergillus niger*. Which help them to survive and compete for nutrients. This plant growth promoting activity were proved by pot experiment in which all the bacteria were shown higher root and shoot length compare to control and maximum root and shoot length was observed by seeds treated with isolate I-25. So this organism was proved to be novel organism for plant growth. I-25 was gram negative, exopolysaccharide producing microorganisms which was lactose non fermenter organism. 16s r-RNA technique for identification of microorganism will proves its molecular identification. So, this organism was proved novel to enhance plant growth by producing various substance.

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