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Isolation of Rhizobacteria from Paddy Field and Their Traits for Plant Growth Promotion

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Abstract

Rice (Oryza sativa L.) is among the three most important cereal crop of the world and its production exceeds from that of wheat. Rhizobacteria has profound effect on plant growth. Present study was carried out to know the effect of rhizobacteria on growth of paddy and specifically the availability of nutrients mainly Nitrogen, Phosphate and Potassium to the Rice. Total 63 such bacteria were isolated on various media from the rhizospheric soil, Ectorhizospheric and Endorhizospheric region along with Bulk soil of paddy field. The isolates were screened in vitro for significant traits viz., qualitative detection of their nitrogen fixing ability, phosphate solubilization and potassium solubilization, Catalase production and enzyme production viz, Protease, Amylase, Lipase Cellulase etc. The plant growth promoting hormones like Indole Acetic Acid (IAA) and Gibberellic Acid (GA) have also been tested quantitatively. Qualitatively nitrogen was fixed (42), phosphate solubilized (57) and potassium solubilized (21). Rhizobacteria also control growth of plant pathogens by producing enzymes like Protease (30), Amylase (30), Lipase (29) and Cellulase (29). Significant amount of Indole Acetic Acid ranging from 5.79 µg/ml to 43.03 µg/ml and Gibberellic Acid ranging from 118.41 µg/ml to 198.18 µg/ml were produced by the isolates. Results indicates that these rhizobacteria may be exploited further for their ability to increase availability of major nutrient and phytohormone supply to the rice and controlling plant pathogens so as to increase productivity of paddy field..

Keywords: Isolation, Rhizobacteria Paddy Field, Traits, Plant Growth Promotion.

Introduction

Rice (*Oryza sativa* L.) is the most important cereal crop of the world. Grain production of rice is exceeded only by that of wheat. With the rapidly increasing world's population there is increasingly important to augment production of cereals, mainly wheat, rice and maize, which accounts for half of the human's calorie intake.

In present situation, plant growth is mainly improved by the application of chemicals that may act as plant growth regulators and as nutrients. Among the nutrients applied to the soil, nitrogen and phosphorous are the major ones. They are added together with potassium, as chemical fertilizers to enhance grain yield. The increased use of chemicals has given rise to several problems such as contamination of water leading to eutrophication, degradation of soil and loss of biodiversity ultimately leading to health risks for humans.

In contrast to these chemicals, there exist beneficial microbes in soil called rhizobacteria which can act as environment friendly alternatives to agrochemicals. Thus their application will increase the sustainability of agriculture. Rhizobacteria are root colonizing bacteria and those which are beneficially influence growth and health of plant through direct or indirect mechanisms are termed as plant growth promoting rhizobacteria[1]. There exists in fertile soil, a huge population of microorganisms which is ubiquitous and at the same time highly proliferating[2]. Rice rhizosphere holds a high diversity of PGPR. It is essential to assess structural and functional diversity for understanding the dynamics of the microbial communities prevailing in rhizosphere[3]. Rhizobacteria can improve plant growth by exploiting varied mechanisms. This may include phytohormone production, nitrogen fixation, phosphate solubilization, resisting plant pathogens etc.[4]. Interactions between the plant and microbes in the rhizosphere are responsible for increasing plant health and soil fertility[5]. Multiple beneficial characteristics possessing strains may provide diverse benefits to the host plant and thus selection of such microbial strains is important present days[6]. Exploring diverse group of rhizobacteria from various crops having useful plant growth promoting traits reduces the cost of fertilizers and at the same time such practices reduces the risk of soil and water pollution from constant use of chemical fertilizers[7]**.** In a chemical-free system, isolation of bacteria is crucial as there are higher chances of bacterial interactions with the rice plant roots. Rhizospheric bacteria, in a less fertilized soil can play more important role particularly in supplying nutrients to the plants[6].

Much work has been done on rice for the isolation of Nitrogen fixing bacteria and/or phosphate solubilizing bacteria. Present research work is an effort to isolate such bacterial flora from paddy field having nitrogen fixing ability, phosphate solubilization and potassium solubilization properties and determining their significant traits which may be helpful for the growth of rice and crop yield.

Material and Methods

Collection of Samples: Soil and rice plants were collected from Paddy fields at Vyara, District-Tapi, Gujarat, India, in summer season during flowering stage. The five rice plants were uprooted from the irrigated plot along with surrounding soil adherent to the roots from the four corners and one from the centre of the plot. The rice plants along with adherent soil and the composite sample of bulk soils were and taken to the laboratory in sterile polythene bags and analyzed within 10-12 hr.

Processing of Sample: 10 gm of soil (rhizospheric/bulk) was weighed and mixed with 90 ml sterile distilled water in 250 ml conical flask. Flask was shaken vigorously for 5 to 10 min to form homogenous suspension. The soil solution than was allowed to settle for 10-15 min before further processing.

For isolation of Ecto-rhizospheric bacteria, roots were washed thoroughly under tap water to remove adherent soil particle and finally, 3 times with distilled water. Then the surface of roots were sampled for isolation.

For isolation of Endo-rhizospheric bacteria, roots were washed thoroughly under tap water to remove adherent soil particle and then surface sterilized by immersing them in 80% ethanol for 3-4 min. Roots were then dipped in sterile 0.05M phosphate buffer, pH 7.0 for 30 min and finally, washed 3 times with distilled water. Roots were then cut into small pieces and macerated in the same buffer using sterilized mortar and pestle.

Isolation of Indigenous Bacteria: Isolation of Nitrogen fixing bacteria: 0.1 ml of supernatant from bulk soil/rhizospheric soil dissolved in sterile distilled water was inoculated into a tube containing Winogradsky medium with Gellan gum as a solidifying agent (autoclaved and cooled to about 50°C)[8]. For isolation of Ectorhizospheric Nitorgen fixing bacteria washed rice roots were placed vertical into a similar tube and for Endorhizospheric Nitorgen fixing bacteria, 0.1 ml of the homogenate obtained by macerating surface sterilized rice roots in sterile phosphate buffer saline, pH 7.0 was transferred into a similar tube. The tube was vortexed and incubated at room temperature (30$\pm$2°C) for 48 hr. The tube with transparent medium showed growth in different sections (viz., top section for aerobic, second for microaerophilic, middle for facultative anaerobe and bottom section for anaerobic nitrogen fixing bacteria) was than broken between two burners and the different sections were cut and transferred to the tubes containing sterile normal saline. A loopful of the culture from these tubes were transferred to various media viz., Ashby's Medium for aerobic nitrogen fixing bacteria, Nfb for microaerophilic, NFM for facultative anaerobe and Winogradsky agar medium for anaerobic nitrogen fixing bacteria (the Winogradsky agar medium for anaerobic nitrogen fixing bacteria were incubated in anaerobic jar). The media plates were then incubated at room temperature (30$\pm$2°C) for 48-72 hr. Morphologically distinct isolated colonies were studied for their growth characteristics and sub-cultured on the respective media for acquiring pure culture of the isolates.

Isolation of Phosphate/Potassium solubilizing bacteria from Bulk/Rhizospheric soil: Using 1 ml supernatant from the bulk/rhizospheric soil suspension, serial dilution was done up to 10^{-6}. 0.1 ml of aliquot from each of the dilutions were transferred and spread on Pikovaskaya's agar medium (for isolation of Phosphate solubilizing bacteria)[9] and on Aleksandrove agar medium (for isolation of Potassium solubilizing bacteria)[10]. The media plates were incubated in incubator at room temperature (30$\pm$2°C) for 48-72 hr. The isolated colonies were studied for their growth characteristics and sub-cultured on the respective media for acquiring pure culture of the isolates.

Determination of Significant Traits: Qualitative nitrogen fixing ability: To test the nitrogen fixing ability, the bacterial cultures were inoculated on Nitrogen free malate medium[11] containing Bromothymol blue as an indicator. The plates were then incubated for 3-4 days at room temperature and were observed for the change in colour of the medium surrounding the colony. For nitrogen fixing bacteria it turns to blue.

Detection of phosphate solubilization: All isolates were screened for phosphate solubilization. A bacterial culture was inoculated on Pikovskaya Agar medium[9] containing inorganic phosphate and then incubated at room temperature. Plates were examined for zone of clearance after 48-72 hrs.

Analysis for qualitative potassium solubilization: Potassium solubilization was checked by inoculating bacterial cultures on Aleksandrove agar medium[10]. Inoculated Aleksandrove agar medium plates were examined for the appearance of zone of clearance around the bacterial growth (indicative of potassium solubilization ability of an isolate) after 5 days of incubation at room temperature.

Catalase production: Catalase production was checked by adding 3% H_2O_2 on the growth of bacteria on solid media. Appearance of effervescences was observed which indicate bacterial ability to produce Catalase. The results were recorded.

Protease production: Protease production of the isolates was verified by means of Skimmed Milk Agar. Fresh bacterial cultures were inoculated as a line and plates were incubated at room temperature for 48hr. Formation of zone of clearance around the line of inoculation is indicative of proteolysis and results were graded as trace, +1, +2, +3 and +4 depending on relative size of zone of clearance[12].

Amylase production: Amylase production was determined by the method described by Alariya *et al*., 2013[13]. Fresh bacterial cultures were inoculated on Starch Agar plate and incubated at room temperature for 24-48hr. Plate was flooded by Iodine

solution after incubation. The hydrolysis of starch is observed as colourless zone around growth with violet background and is reported as trace to 4+.

Lipase production: Lipase production was checked using Tributyrene Agar medium. Lipolytic activity is recorded by observing formation of zone of clearance around the line of inoculation after the plates were incubated at room temperature for 3 days. Results were reported as trace to +4 based on relative size of clearance zone[14].

Cellulase production: For determination of Cellulase production the method described by Aneja[15] was employed. Fresh bacterial cultures were inoculated on Modified Czapek Mineral salt medium and after incubation at room temperature (30±2°C) for 5 days, media were flooded with 1% solution of Congo red for 30 min, followed by decolourization with Normal Saline. Intact cellulose binds with Congo red and will not be decolorized, while the degraded part will be decolorized and was observed as a zone of clearence. Results were observed and reported as trace to +4 considering comparative zone of clearance.

Pectinase activity: Pectinase production for selected isolates was determined by following the method as mentioned by Aneja[15]. For which, line inoculation was done using fresh bacterial cultures on Hankin's agar medium. After incubation for 3-5 days at room temperature (30±2°C), 1% aqueous solution of Hexadecyl trimethyl ammonium bromide was poured on medium and observed for the formation of zone of clearance around the line of inoculation which indicates pectinase production. Results were as gradation from the zone size.

Quantitative estimation of Indole Acetic Acid production: Estimation of Indole Acetic Acid production was determined by the method of Gorden and Paleg[16]. The fresh bacterial cultures were inoculated in 50 ml Nutrient broth (in 100ml conical flask) containing L-tryptophan (5µg/ml) and incubated at room temperature (30±2°C) for 7 days. After incubation broth cultures were centrifuged at 8000 rpm for 5 min. To 2ml supernatant, 2 drops of O-phosphoric acid was added followed by addition of 4ml of Salkowaski reagent (50ml, 35% perchloric acid and 1ml 0.5M $FeCl_3$). The absorbance of reaction mixture was measured at 535nm after incubation in dark for 30min. The quantitation of IAA production was done by extrapolating the absorbance values of test solutions with standard graph prepared using varying concentrations of standard IAA and were expressed as µg/ml.

Quantitative estimation of Gibberellic Acid production: Gibberellic acid production was quantitatively checked by following the method of Borrow et al., 1955[17]. The fresh bacterial cultures were inoculated in 50 ml Nutrient broth (in 100 ml conical flask) and incubated at room temperature (30±2°C) for 7 days. After incubation broth cultures were centrifuged at 8000 rpm for 5 min. To 15ml of supernatant transferred to another tube 2ml of zinc acetate solution (21.9gm Zinc acetate was dissolved in 80ml distilled water and 1ml of Glacial Acetic acid was added, mixed well and final volume was set to 100 ml) was added. After 2 min. 2ml of potassium ferrocyanide solution was added. Solution was mixed properly and then centrifuged at 8000 rpm for 10 min. To the 2ml supernatant, 2ml of 30% HCl was added and incubated for 75 min at room temperature. Absorbance was measured at 254nm against 5% HCl as blank. The concentration of GA was determined by extrapolating the absorbance values of test solutions with standard graph prepared using varying concentrations of standard GA and were expressed as µg/ml.

Results and Discussion

Isolation of bacteria from soil and roots: Total 63 bacteria were isolated on different media from rhizospheric soil, ectorhizospheric region, endorhizospheric region as well as bulk soil. The number of isolates obtained on different media is mentioned in table-1.

18 phosphate solubilizing bacteria (PSB), 12 potassium solubilizing bacteria (KSB) and 33 nitrogen fixing bacteria (NFB) were successfully isolated in present study. 14 PSB were isolated from rhizosphere soil compared to 4 isolates from bulk soil. Our results are in agreement with the report of Reyes *et al*.[18] who have mentioned that rhizospheric soil possesses higher concentrations of phosphates solubilizing bacteria as compared to non-rhizosphere soil.

Multi-trait positive Isolates: Results of the present work revealed that out of 63, all the isolates have represented at least 3 plant growth promoting traits. As presented in table-2, 27 isolates that have shown multi-trait positivity for qualitative tests and thus are significant PGPR. Out of total isolates, highest multi-trait (maximum eight out of eight qualitative traits checked) found in 5 isolates, followed by 11 isolates that showed seven positive traits and another 11 isolates have six positive traits.

Plant growth promoting bacteria influences plant growth through direct or indirect mechanism. The direct mechanisms include either production of plant growth promoting substances or by facilitating uptake of nutrients from the environment. Indirect mechanisms include minimizing or preventing deleterious effect of some of the phytopathogenic microorganisms and aiding the plant to resist environmental stress[19]. There were 42 isolates showing Nitrogen fixing ability. Significant Phosphate solubilization was detected for many isolates (57) ranging from trace to 4+ grade; four isolates recorded 4+ grade and ten up to 3+ grade. Potassium solubilizing bacteria may increase release of potassium from potassium bearing minerals like mica, through production of acids, alkalis or chelating compounds[6]. Potassium solubilization was detected in the range from trace to 2+ for 18 isolates in the present work.

Table-1
Number of Isolates obtained on different Media and from different sections

Isolation Medium	Number of Isolates				Total Isolates
	Rhizospheric Soil	Bulk Soil	Ectorhizospheric Region	Endorhizospheric Region	
Pikovaskaya	14	4	-	-	18
Aleksandrove	9	3	-	-	12
Ashby Agar	4	4	3	2	13
Nitrogen Free Malate Medium	3	7	6	1	17
Nfb Agar Medium	-	-	1	2	3

Table-2
Plant Growth promoting traits of Multi-trait positive Isolates

S. No.	Isolate No	Nitrogen Fixing ability	Phosphate Solubilization	Potassium Solubilization	Amylase Production	Protease Production	Lipase Production	Cellulase Production	Catalase Test	Total Traits positive
1	RS/PK/01	+	+++	-	++++	++++	++++	++++	+	7
2	RS/PK/02	+	+++	-	+	+++	++++	++++	+	7
3	RS/PK/03	+	++	Trace	+++	Trace	++++	+++	+	8
4	RS/PK/04	+	++	-	+++	+++	+++	-	+	6
5	RS/PK/05	+	++++	++	++++	+	+++	++++	+	8
6	RS/PK/06	+	+++	++	Trace	++++	-	+++	+	7
7	RS/PK/07	+	++++	Trace	-	+++	+++	+++	+	7
8	RS/PK/08	+	+++	-	Trace	-	+	Trace	+	6
9	RS/PK/09	+	+++	+	++++	++++	++	++	+	8
10	RS/PK/10	+	+++	Trace	++	-	-	++	+	6
11	RS/PK/11	+	+	-	+++	+++	-	Trace	+	6
12	RS/PK/12	+	++++	-	++++	+++	Trace	+++	+	7
13	RS/PK/13	+	+++	-	-	+++	++	+++	+	6
14	RS/PK/14	+	+++	Trace	+	+++	-	-	+	6
15	BS/PK/01	+	++++	-	++	++	+++	+++	+	7
16	BS/PK/03	+	++	-	+++	++++	-	++++	+	6
17	BS/PK/04	+	+++	-	+++	++++	-	+	+	6
18	EC/NFM/03	+	+	+	++++	++++	+	++++	+	8
19	EC/NFM/06	+	++	-	+	++++	++	+++	+	7
20	RS/NFM/03	+	+	-	++	++	-	++	+	6
21	BS/NFM/03	+	+	-	++++	++++	-	+++	+	6
22	BS/NFM/04	+	+	+	++++	++++	+	Trace	+	8
23	EN/NFB/01	+	++	-	Trace	++++	+++	++	+	7
24	EC/NFB/01	+	++	-	-	++++	++	Trace	+	6
25	EC/ASH/02	+	Trace	-	++++	++++	++	Trace	+	7
26	RS/ALK/02	+	+	Trace	++	+++	++	-	+	7
27	RS/ALK/03	+	++	Trace	++	++	+	-	+	7

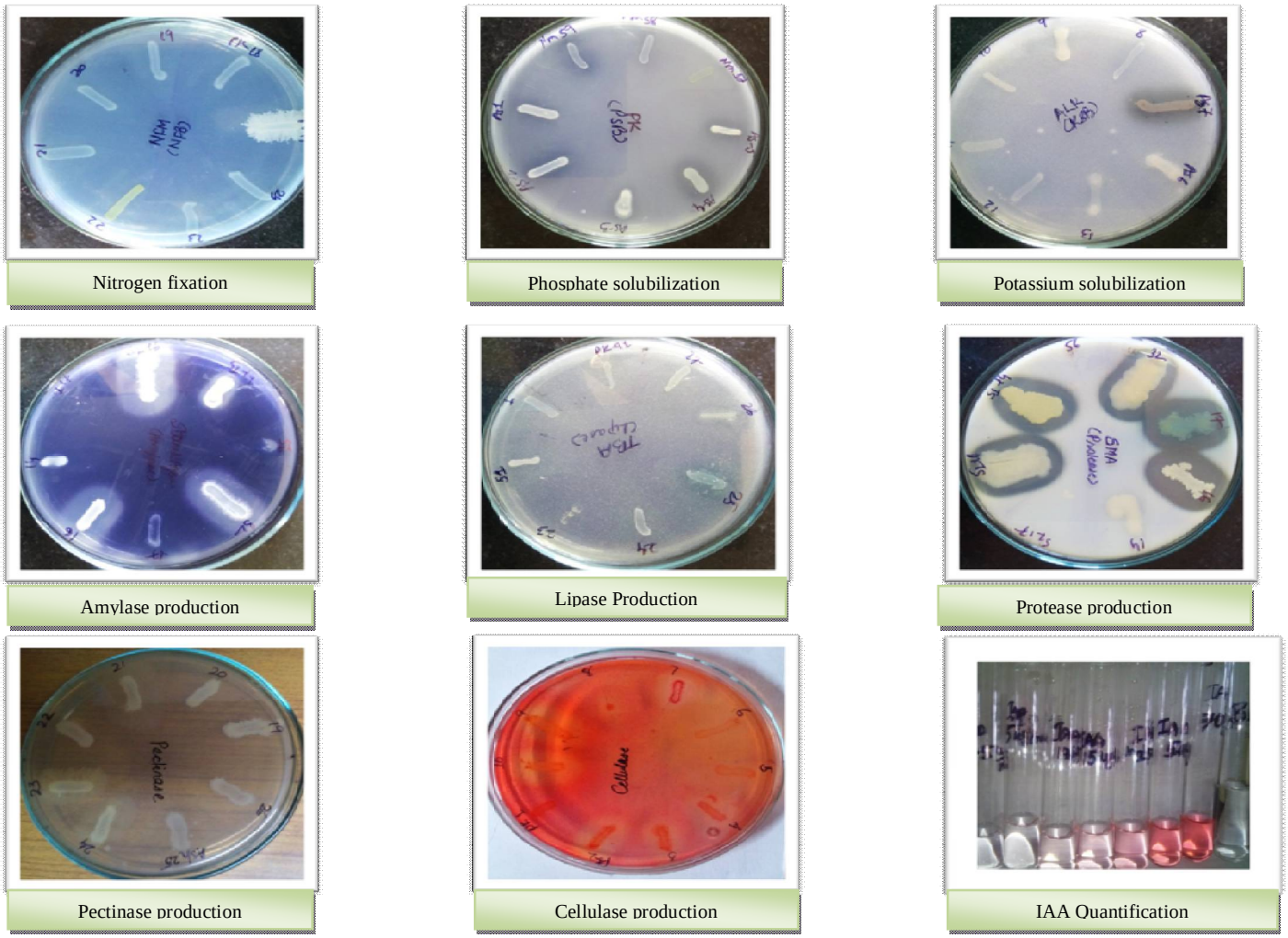

Figure-1
PGP traits of isolates

Among indirect traits that help to control growth of plant pathogens are production of enzymes like protease, amylase, lipase and catalase as well as ammonia and HCN production. Excellent performance, graded 2+ to 4+ for many isolates have been reported viz., 26 isolates for Protease, 22 for Amylase and 19 for Lipase. Earlier reports proposes that production of hydrolyzing enzyme, chiefly cellulase and pectinase, could permit the bacteria to penetrate into roots and thus they will remain as endophytes, since cell wall is mainly composed of cellulose whereas the middle lamella part between cell walls largely contains pectin[6]. In accordance with the same, in present study 20 isolates out of total 63 were reported positive for cellulase production and 13 out of total 15 selectively tested isolates represented pectinase production. Catalase production has been reported as important traits of PGPR isolates that indirectly influence the plant growth. Catalase production was exhibited by all the isolates, the finding is in accordance with the report and it also indicates that all isolates are aerobic in nature. Catalase producing rhizobacteria must be highly resistant to abiotic stress viz., environmental, mechanical and chemical[20].

Quantitation of Growth Hormones: IAA and GA production are considered to be important plant growth promoting features of rhizospheric isolates. IAA is involved in numerous physiological processes of plant viz., cell division and differentiation, stimulation of seed germination, xylem and root development, lateral and adventitious root formation, photosynthesis, pigment formation, various metabolites synthesis and resistance to stressful conditions etc.[21-23]. Gibberellins influence various plant developmental and physiological processes[24,25]. Seed germination, emergence of seedling, stem and leaf growth, floral induction and growth of flower and fruit are some of these processes[26-28]. In the present study, all the isolates were tested for their plant growth hormone i.e. Indole acetic acid and Gibberellic acid producing ability. All the 63 isolates were able to produce IAA and GA to differing extent. The IAA and GA production by the isolates ranges from 5.79µg/ml to 43.03µg/ml and 118.41µg/ml 198.18µg/ml

respectively as has been shown in table-3 and represented by Figure-2 and Figure-3. Many isolates were promising for the growth hormone producing ability in in-vitro condition and thus can be checked further by pot and the field experiments for their effect on physiological traits of rice plant as has been reported by many researchers.

Table-3
IAA and GA production by the isolates:

Isolate No	IAA in µg/ml	GA in µg/ml	Isolate No	IAA in µg/ml	GA in µg/ml	Isolate No	IAA in µg/ml	GA in µg/ml
RS/PK/01	5.79	133.98	EC/NFM/03	11.71	136.59	EC/ASH/03	32.24	139.20
RS/PK/02	13.95	173.86	EC/NFM/04	8.03	147.16	RS/ASH/01	29.74	123.86
RS/PK/03	7.11	118.41	EC/NFM/05	10.13	125.57	RS/ASH/02	36.45	137.73
RS/PK/04	8.42	156.36	EC/NFM/06	20.39	128.30	RS/ASH/03	30.66	132.39
RS/PK/05	10.13	130.00	RS/NFM/01	10.39	129.55	RS/ASH/04	34.87	141.02
RS/PK/06	10.26	138.98	RS/NFM/02	14.21	131.70	BS/ASH/01	12.63	124.43
RS/PK/07	15.79	154.55	RS/NFM/03	14.08	123.75	BS/ASH/02	43.03	167.16
RS/PK/08	11.05	143.18	BS/NFM/01	31.45	136.02	BS/ASH/03	32.63	158.52
RS/PK/09	26.97	174.20	BS/NFM/02	32.89	145.68	BS/ASH/04	22.89	161.02
RS/PK/10	14.08	193.30	BS/NFM/03	13.68	140.91	RS/ALK/01	12.89	98.86
RS/PK/11	35.79	128.18	BS/NFM/04	12.37	119.66	RS/ALK/02	10.00	107.95
RS/PK/12	19.74	133.07	BS/NFM/05	12.63	126.70	RS/ALK/03	12.89	101.14
RS/PK/13	20.53	130.68	BS/NFM/06	14.08	137.16	RS/ALK/04	17.63	189.21
RS/PK/14	15.79	125.34	BS/NFM/07	13.82	137.27	RS/ALK/05	9.47	98.86
BS/PK/01	9.21	147.73	EN/NFB/01	30.66	198.07	RS/ALK/06	11.18	111.36
BS/PK/02	16.32	141.25	EN/NFB/02	32.89	190.11	RS/ALK/07	9.61	97.73
BS/PK/03	7.37	137.61	EC/NFB/01	38.82	183.30	RS/ALK/08	8.55	107.95
BS/PK/04	19.08	168.64	EN/ASH/01	38.03	140.57	RS/ALK/09	27.11	188.98
EN/NFM/01	33.16	142.16	EN/ASH/02	11.05	124.20	BS/ALK/01	12.24	90.91
EC/NFM/01	33.95	141.59	EC/ASH/01	15.00	127.84	BS/ALK/02	7.76	98.86
EC/NFM/02	34.34	145.45	EC/ASH/02	7.89	147.39	BS/ALK/03	24.08	198.18

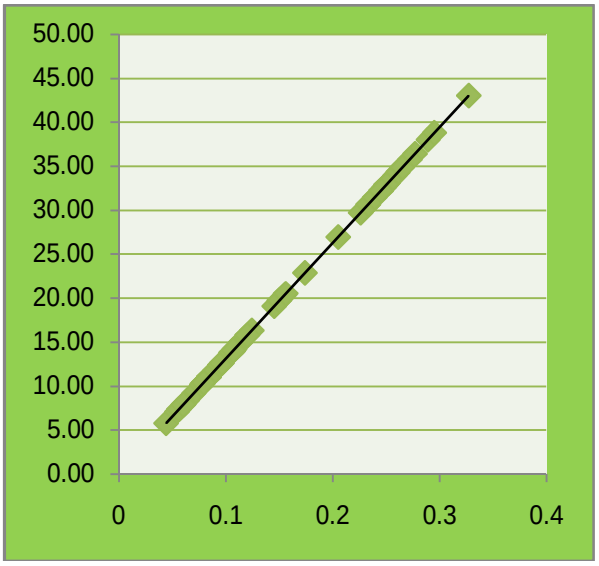

Figure-2
Quantitation of IAA

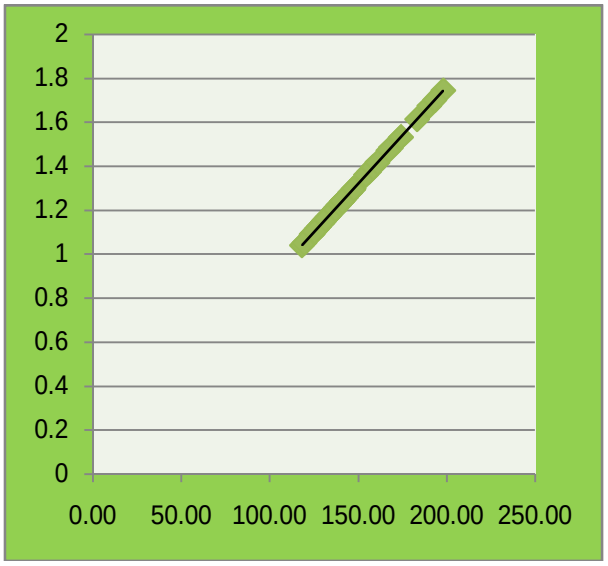

Figure-3
Quantitation of GA

Conclusion

The qualitative trait results indicated that RS/PK/03, RS/PK/05, RS/PK/09, EC/NFM/03 and BS/NFM/04 were found to be most efficient isolates. These isolates have also showed relatively good IAA and GA production. They can be further checked by field experiment for their plant growth promoting effect on paddy crop. Applying such efficient PGPR singly or in consortium as bioinoculants can be an approach to replace use of chemicals as fertilizers and pesticides for sustainable cultivation of rice in India and globally as well[29].

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