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Screening and Identification of traits in plant growth promoting rhizobacteria from rhizospheric soils of *Persea bombycina*

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Abstract: *The rhizosphere is the region of soil that is influenced by root secretions and associated soil microorganisms. Plant growth promotingrhizobacteria (PGPR) are a heterogeneous group of bacteria found in the rhizosphere . Here, an attempt has been made to screen and identify PGPR traits in bacteria isolated and characterized from Perseabombycinarhizospheric soils. Samples were collected from rhizosphere of Som (Perseabombycina) growing in the Regional Botanical Garden, Department of Botany, Gauhati University, Guwahati. 14 pure cultures were obtained in solid Nutient Agar Media. For in vitro screening of isolates for their PGP activities, tests include Indole Acetic Acid Test, quantitative analysis of IAA, production of ammonia (NH_3), production of hydrogen cyanide (HCN). Isolates were screened on Pikovskaya's agar plates for phosphate solubilization. The test isolates gave a positive result towards Gram's staining reaction. The 14 isolates were able to produce IAA as well as positive for Phosphate solubilization and Ammonia Production whereas negative for Cyanide production. With the addition of tryptophan from 50 to 500 µg/ml the production of IAA was increased. This study will facilitate accurate identification of the bacterial types that harbour PGPR traits essential in combating foliar fungal pathogens of Perseabombycina and in sight an immunological response in the plants (Perseabombycina), thereby enhancing their capability to overcome fungal diseases.*

Keywords: *nitrogen fixation, Perseabombycina,plant growth promoting rhizobacteria, rhizosphere*

I. Introduction

The rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms. The rhizosphere contains many bacteria that feed on sloughed-off plant cells, termed as*rhizodeposition*. Protozoa and nematodes that graze on bacteria are also more abundant in the rhizosphere. Thus, much of the nutrient cycling and disease suppression needed by plants occurs immediately adjacent to roots.Among the free living nitrogen fixing bacteria Azospirillum is considered to be more efficient with nitrogenase properties comparatively better than the other nitrogen fixers. Nitrogen fixation is the first major mechanism for the enhancement of plant growth by Azospirillum [18].

The nitrogen fixing efficiency of Azospirillum species has been examined by several workers. Observed 2.4 to 18.28 mg of N per gram of carbon source utilized by 25 Azospirillum strains isolated from 14 genotypes of cotton [19]. A. lipoferum and A. brasilense showed nitrogen fixation in the range of 7.54 to 24.53 mg of N per gram of malic acid after seven days at 280C under static condition [27]. Several heterotrophic and chaemoautotrophic bacteria, fungi and actinomycetes have the capacity to solubilize insoluble mineral phosphates. Reported B. polymyxa and X. maltophila isolated from field grown plants to solubilize Carolina rock phosphate to the extent of 19.9 per cent and 22.5 per cent respectively [7]. Recorded solubilization of 11 to 72 per cent TCP in broth medium by pseudomonads isolated from rhizosphere and non-rhizosphere soils [5]. Siderophores are low molecular weight iron-binding ligands which can bind to ferric ion and make it available to the producer microorganism [16]. Pyoverdin type siderophores from P. aeruginosa have shown to exhibit both antifungal and antibacterial activity [12]. [20] observed disease suppression and enhanced growth and nodulation of lentils due to siderophore mediated biocontrol activity of fluorescent pseudomonads in Fusarium infested soil. In vitro suppression of plant pathogens by siderophores of fluorescent pseudomonads was also reported by [15]. [28] reported production of siderophores as one of the mechanisms for in vitro inhibition of Colletotrichumdematium, Rhizoctoniasolani and Sclerotiumrolfsii by fluorescent pseudomonads. [26] observed that siderophores produced by Pseudomonas fluorescens were antagonistic to fungal pathogens like Fusariumoxysporum, Alternaria sp. and Colletotrichumcapsicii. Production of iron chelating hydroxymate type siderophores by P. fluorescens NCIM 5096 and P. putida NCIM 2847 was reported by [24]. HCN inhibits the

electron transport thereby the energy supply to the cell is disrupted leading to the death of the organism. It inhibits proper functioning of enzymes and natural receptors by reversible mechanism of inhibition [4]. It is also known to inhibit the action of cytochrome oxidase [9]. HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens [6]. Fluorescent Pseudomonas strain RRS, isolated from Rajanigandha, a flowering plant produced HCN and the strain improved seed germination and root length [23]. Production of HCN by Pseudomonas strain EM 85 was reported by [1]. [11] reported that approximately 32 per cent of bacteria from a collection of over 2000 isolates were cyanogenic, evolving HCN from trace concentration to >30 n moles/mg cellular protein. They also opined that cyanogenesis was predominantly associated with pseudomonads and was enhanced when glycine was provided in the culture medium. [20] reported that HCN synthase is encoded by three biosynthetic genes (hcn A, hcn B and hcn C) in fluorescent pseudomonads. [17] studied antagonistic mechanisms of fluorescent pseudomonads against Phytohthoracapsicii in black pepper and reported many isolates to produce HCN which limited the growth of P. capsicii. Ten fluorescent pseudomonads isolated from rhizosphere of sunflower, potato, maize and groundnut were shown to enhance plant growth and suppress the collar rot of sunflower where in one of the mechanism of suppression of the disease was by HCN production [25]. Pseudomonas populations producing HCN were reported in the rhizosphere of tobacco in Swiss soils suppressive to Thielaviopsisbasicola, causal agent of black root rot of tobacco by [21].

In the present study, an attempt has been made to screen and identify Plant Growth Promoting Rhizobacteria traits in bacteria isolated and characterized from Perseabombycinarhizospheric soils. The present study will facilitate accurate identification of the bacterial types that harbour Plant Growth Promoting Rhizobacteria traits essential in combating foliar fungal pathogens of Perseabombycina and in sight an immunological response in the plants (Perseabombycina), thereby enhancing their capability to overcome fungal diseases.

II. Materials And Methods:

The present study was carried out at the Department of Biotechnology, Gauhati University. The materials used and the method followed is as described:

2.1 Collection of Rhizospheric soil

For the present study soil samples were collected from rhizosphere of Som (P*erseabombycina*) growing in the Regional Botanical Garden, Department of Botany, Gauhati University, Guwahati -781014, in sterile polythene bags and carried to laboratory. The collected soil samples were air dried in shade for 4-5 days.

2.2Isolation and characterization of bacteria inhabitatingrhizosphere of Som (Perseabombycina)

One gram of the processed soil was weighed and dissolved into 10 ml of double distilled water and diluted 1000 folds by serial dilution method, for successful isolation pure cultures by repetitive streaking in solid Nutrient Agar medium (Peptone 5g, NaCl 5g, Beef extract 1.5g, Yeast extract 1.5g and Agar 15g per litre). The petri-plates were incubated at 28 ^{0}C and after 5 successive streaking of the original consortium for a number of batches; pure cultures of single colony isolates were obtained which were identified in a Labomed ATC-2000 microscope under 1000 x. Altogether 14 different bacterial isolates from the collected starter culture materials were selected and identified through microscopic observation which were inoculated into Nurient broth medium and incubated at 30^0 C to obtain mass culture of the isolates.

2.2 Identification and Characterization:

The bacterial isolates were characterized by their cultural conditions, morphological and biochemical characteristics using standard methods :

2.2.1Morphological identification:

Morphological identification of the bacterial strains was done on the basis of Gram's Staining.

2.2.2Invitro screening of bacterial isolates for their plant growth promoting (PGP) activities :

2.2.2.1 Indoleacetic acid test:

Test for indoleacetic acid (IAA) production or IAA production was performed by following the modified method as described by Brick et al. (1991) [2]. Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986) [13] at different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 mg/ml). Bacterial cultures were inoculated and grown for 48-72 hrs on nutrient broth at 30°C. 10µl culture was inoculated in 20m broth. Fully grown cultures were vortexed and centrifuged at 7000 rpm for 15 mins at 4°C. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent (50

ml, 35% of perchloric acid, 1ml 0.5M FeCl3 solution). Development of pink colour indicates IAA production. Optical density was taken at 530, 535 and 540nm with the help of spectrophotometer.

2.2.2.2 NH3 production:

Production of ammonia by the bacterial isolates considered in our study was tested in peptone water. Freshly grown cultures in nutrient broth for 48-72hrs in 30°C were inoculated in 10ml peptone water in each tube and incubated for 48–72 h at 28°C. Nessler'sreagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992) [3].

2.2.2.3 HCN production:

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck (1948) [14]. Briefly, nutrient broth was amended with 4.4 g glycine/l and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28°C for 4 days. Development of orange to red colour indicated HCN production

2.2.2.4 Phosphate solubilization by test bacteria:

All isolates were first screened on Pikovskaya's agar plates for phosphate solubilization as described by Gaur (1990) [8]. Quantitative analysis of solubilization of tricalcium phosphate in liquid medium was made as described by King (1932). Briefly, the test isolates were inoculated in 25 ml Pikovskaya's broth and incubated for 4 days at 28°C. The bacterial cultures were centrifuged at 15,000 rpm for 30 min. Supernatant (1 ml) was mixed with 10 ml of chloromolibidic acid and the volume was made up to 45 ml with distilled water. Cholorostannous acid (0.25 ml) was added and the volume was made up to 50 ml with distilled water. The absorbance of the developing blue colour was read at 600 nm.

III. Results And Discussion

The results of the experiments carried out to screen Plant Growth Promoting Traits of the 14 PGPR test isolates are presented under the following headings :

3.1 Identification of PGPR:

All the 14 efficient PGPR strain were tested for gram's staining to identify their morphological characteristics. All the test isolates gave a positive result towards gram's staining reaction conferring them as gram positive strain. The results obtained are depicted in the TABLE 1 and shown in figure 1.

3.2Plant growth promoting traits of test isolates in-vitro:

Results obtained from the present study showed that all the 14 isolates of PGPR are able to produce IAA. Phosphate solubilization was detected in all the 14 isolates of PGPR. Moreover, the test isolates gave a positive result for ammonia production whereas negative for Cyanide production.

3.2.1 Quantitative assay of IAA production by selected isolates:

All the isolates were tested for the quantitative estimation of IAA in the presence of different concentrations of tryptophan. With no addition of tryptophan, production of IAA was not observed. With the addition of tryptophan from 50 to 500 µg/ml the production of IAA was increased. The production of IAA was highest in isolates of 2P. Minimum amount of IAA production was detected in the isolate of A/11. The concentrations of tryptophan for each isolate is depicted in TABLE 2 and shown in figure 2.

Table 2: Concentrations of Tryptophan for each isolate

Isolate name	concentration of Tryptophan				
	0	50	150	300	500
103/3	46	80	64	8	12
103/7	30	52	26.7	18	14
105/3	18	52	90	26	18
105/5	24	50	39	52	12
107/4	10	88	44.7	172	30
A/11	2	12	24	4	10
A2/1	48	53	29	66	110
A2/4	10	60	56	100	66
A2/5	30	76	17	6	14
1P	24	46	38	42	24
2P	22	60	57	70	142
3P	18	66	46	3	68

5P	12	64	56	96	36
12P	18	28	70	104	96

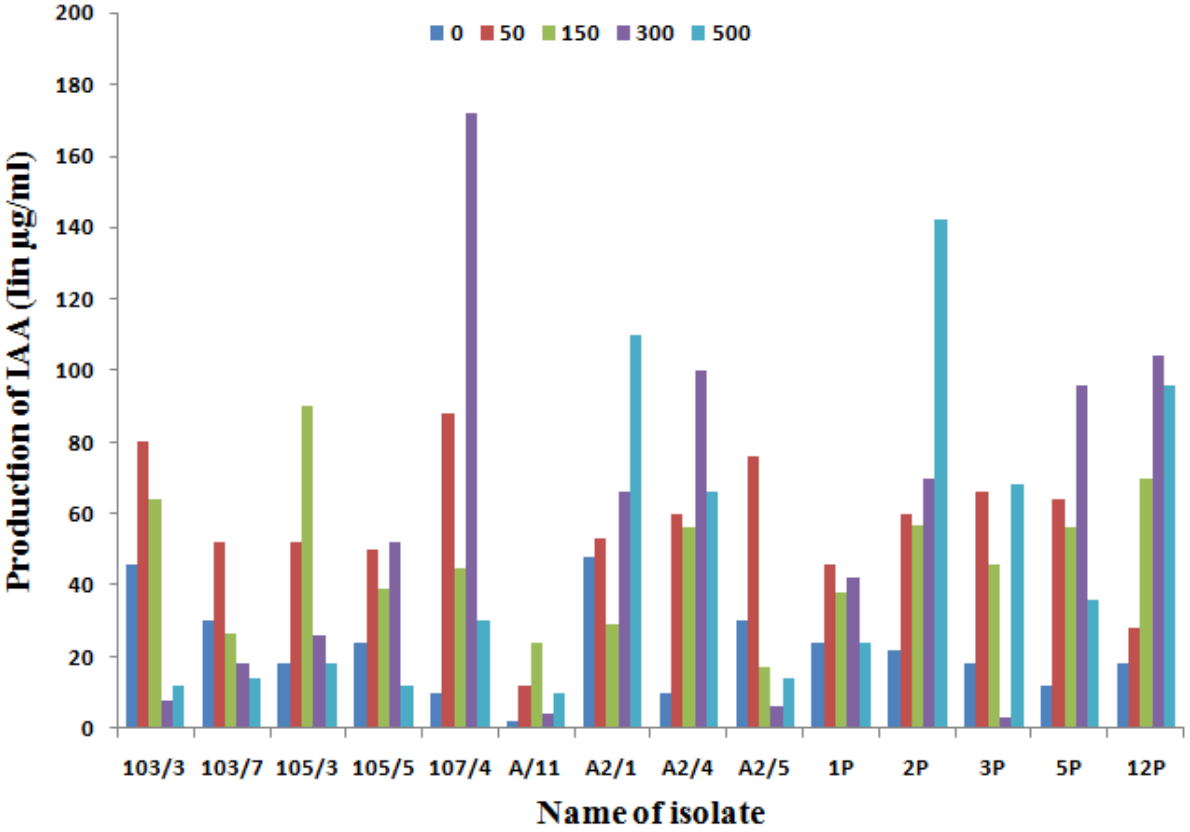

Fig. 2: production of iaa/per isolate

3.2.2 Analysis of phosphorus solubilizing activity by isolates:

All the test isolates were screened for phosphate solubilization activity using Pikovskaya's Broth medium. Results obtained from the study showed phosphate solubilizing activity of all the test isolates. However, some isolates showed the lowest values (Table-3). The results were summarized in the table-3 and shown in Figure 3 and 4.

Table 3: Phosphorus solubilization by test isolates

Isolate Name	Phosphate solubilization (µg/50ml)
103/3	65.91
103/7	102.04
105/3	261.81
105/5	151.59
107/4	135.91
A/11	24.54
A2/1	124.09
A2/4	189.77
A2/5	14.77
1P	162.27
2P	172.72
3P	172.95
5P	218.64
12P	173.86

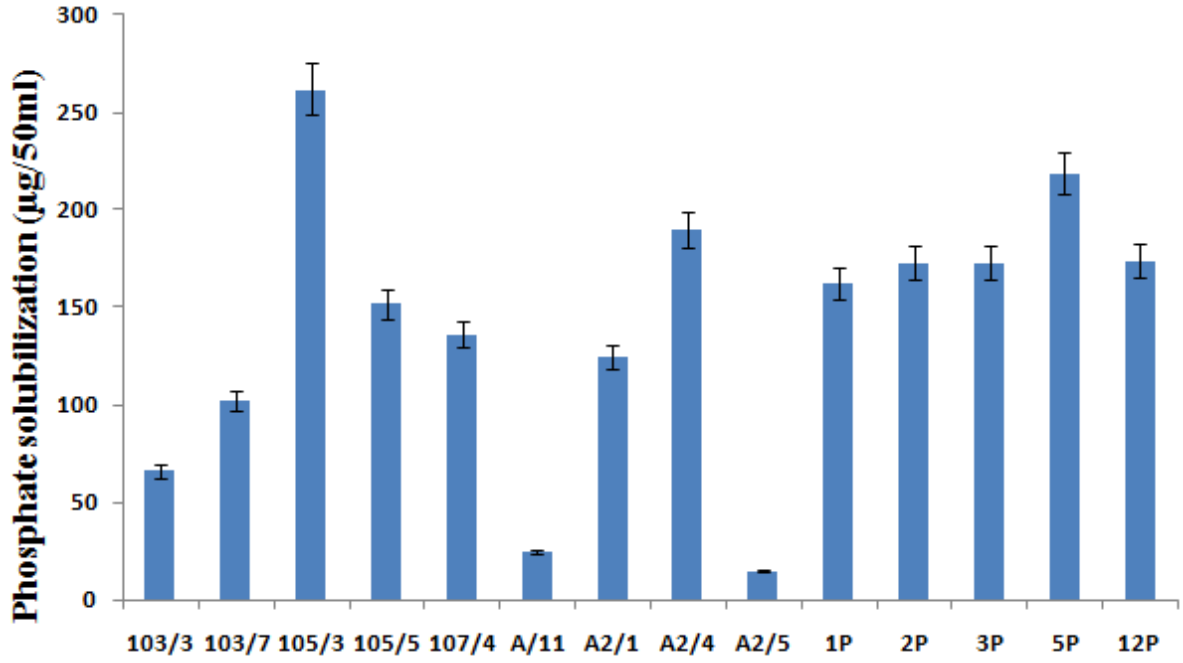

Fig. 3: phosphate solubilization/isolate

3.2.3 Production of ammonia:

All the tested PGPR strain showed positive result for ammonia production. The isolates incubated in peptone water developed brown to yellow colour when Nessler,s reagent was added, indicating a positive test for ammonia production. Result as shown in figure 5.

3.2.4 Hydrogen Cyanide production:

Screening of PGPR strain for Cyanide production activity gave a negative result typical for plant growth promoting rhizobacteria as shown in figure 6.

IV. Conclusion

Plant rhizosphere is known to be preferred ecological niche for soil microorganisms due to rich nutrient availability. It has been assumed that inoculation with diazotrophic bacteria like Rhizobium, Azotobacter and Azospirillum enhanced the plant growth as result of their ability to fix nitrogen. However, despite of extensive research efforts only rhizobia have been shown to increase yields from dinitrogen fixation. Growth promotion may be attributed to other machanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities. Reports are also available on Azotobacter spp. isolated from different sources showed IAA production.

In the present investigation 14 isolates of Plant Growth Promoting Rhizobacteria, isolated from rhizospheric soils of *Perseabombycena* were screened in vitro for PGP activities.

4.1 Morphology study:

In morphology study, all the test isolates were found as gram positive in Gram's staining reaction.

4.2 Indole acetic acid production :

IAA production was detected in all the isolates at varying level at different concentrations of precursor tryptophan. There was a increase in the level of IAA with the increase in concentration of tryptophan (50-500micro gram per ml) This helps in the induction of longer roots of plants with number of root hairs and root laterals. This improves nutrient uptake efficiency of plants. IAA also causes cell enlargement, cell division, phototropism, geotropism and apical dominance in plants. These mechanisms help in the promotion of plant growth. In other words IAA production was detected in all the 14 isolates. The ability of bacteria to produce IAA in their rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. It was observed that with the addition of tryptophan from 50 to 500µg/ml the production of IAA was increased. Similar trend of IAA production with the increasing concentration of tryptophan was also reported by some workers. In addition, the production ability of IAA in among rhizobial strains is not the same. Highest amount of IAA production was observed in the isolate 2P with 22, 60, 57,70, and 142 µg/ml at different tryptophan

concentration(0, 50, 150, 300, 400, 500) respectively. The PGPR isolate found to be lowest producer of tryptophan was A/11. Amount of IAA production by the isolate A/11 was detected as 2, 12, 24, 4, 10 µg/ml with respect to Tryptophan concentration at 0, 50,150, 300, 400 and 500 µg/ml

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4.3 Phosphate solubilization:

Phosphorus is the second required mineral nutrient for plants. In the present study most of the test isolates showed phosphate solubilization. Phosphorus limits the growth of terrestrial plants. Soil contains large reserves of phosphorus but only tiny amount remain in available form for plants. Plants only can absorb P in two soluble forms, the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) ions. Rhizobacteria converts this immobilized form of P into available form for the plants. Rhizobacteria increases nutrient availability to host plants.

Phosphate solubilization was frequently encountered by all the test isolates.In the present study, among all the isolates, 105/3 was found to be the best phosphate solubilizer with the phosphate solubilizing activity of 261.81µg/50ml. Minimun phosphate solubilizing activity was observed in A2/5, withthe phosphate solubilizing activity of 14.77µg/50ml.

4.4 Ammonia (NH_3) production:

Ammonia production was found as a common trait in all the selected groups of bacteria. Nitrogen is a primary requirement for the plant growth and development. Rhizobacteria fixes atmospheric nitrogen and converts it to ammonia to make it available for the plants. In other words, all the 14 strains showed positive test for NH_3 production.

4.5 HCN production:

All the 14 strains were found negative for HCN production.

The findings of the present studies suggest that all the test isolates of PGPR have potential plant growth promoting traits. Thus the present investigation, "Screening and Identification of Plant Growth Promoting Traits in Plant Growth Promoting rhizobacteria from rhizospheric soils of *Perseabombycina"* has thrown some light into the PGP traits of the PGPR for their effective and beneficial application in plants to promote growth and provide protection against diseases. However, further research is required in this area to screen out for efficient strain of PGPR.

V. Figures

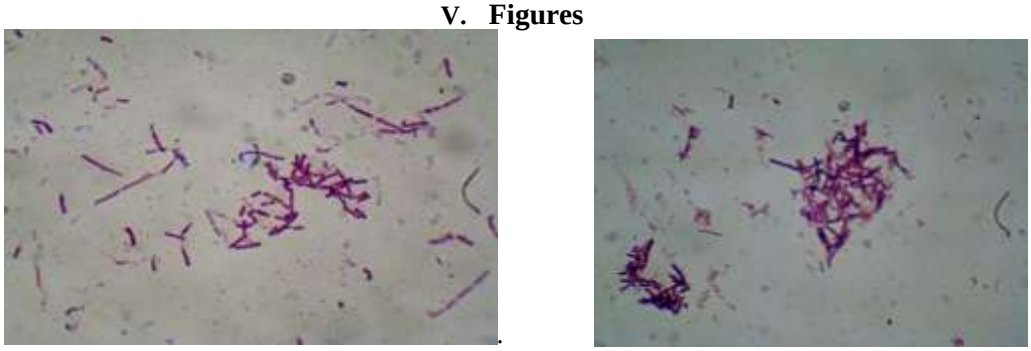

Fig. 1 gram's staining of test isolates

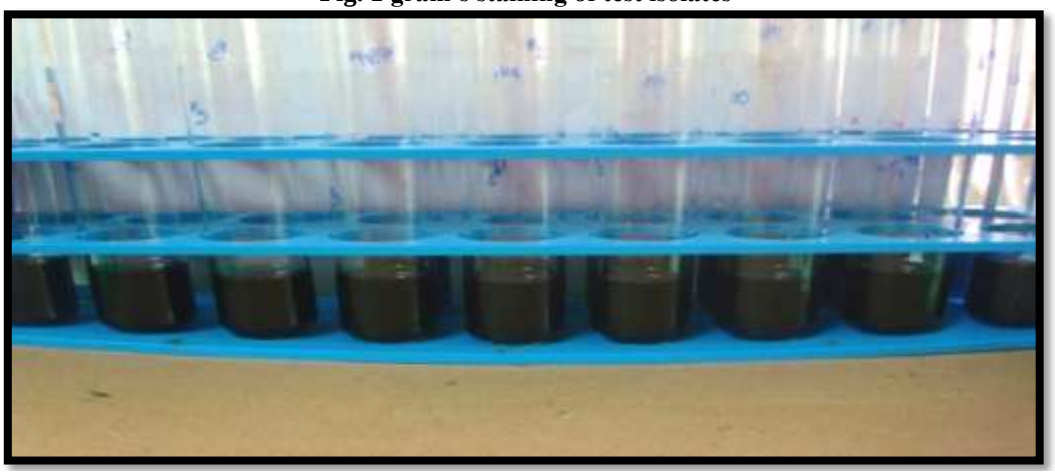

fig. 4 phosphorus solubilization by test isolates

Fig. 5 production of ammonia by test isolates

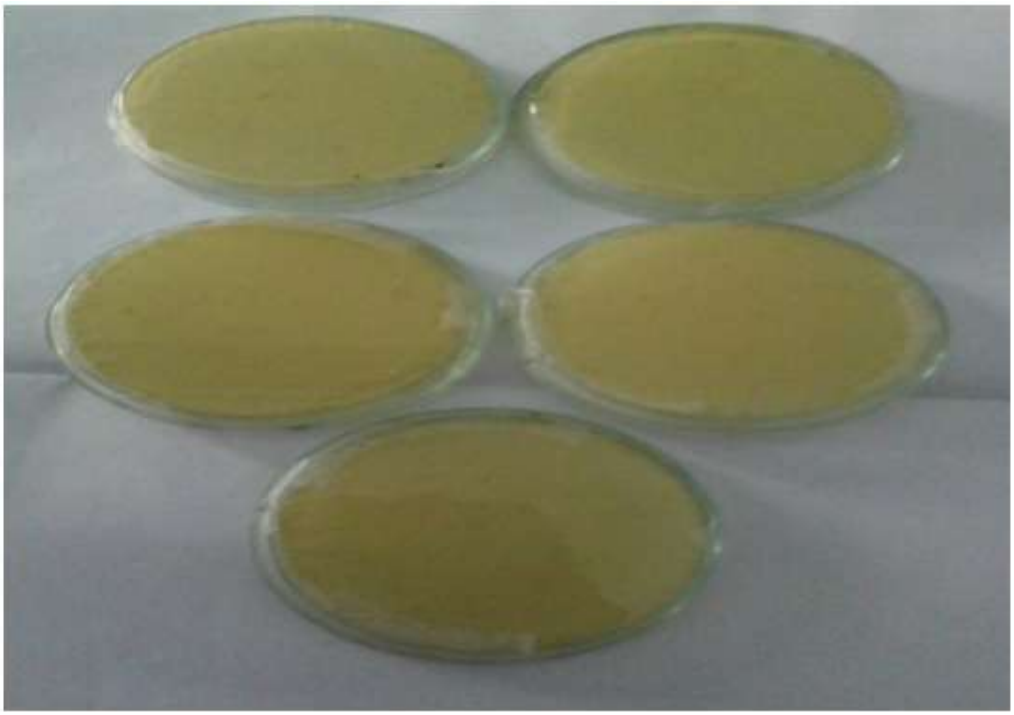

Fig. 6 production of hcn by test isolates

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