Complementation of biochemical and physiological assays with functional PGPR based assays to screen potential isolates

Abstract:

The work deals with the biochemical characterization of rhizospheric isolates including Indole, Methyl Red, Voges Proskauer, Citrate test (IMViC test) based quantitative biochemical assay, PGPR properties like nitrogen assimilation (in microbes), phosphate solubilization etc. A special aspect of finding the correlation between the biochemical tests and PGPR properties were also studied. Methyl red test and tryptophanase assay (by indole test) were found to be positively and negatively correlated with phosphate solubilization. Vogues Proskeur and citrate utilization tests were negatively correlated with the phosphate solubilization. In future, these biochemical tests can be used as determining factors to identify phosphate solubilizing bacteria. Moreover, a group of bacteria was identified by the scatter plot analysis which shows low acid production with high phosphate solubilization. Lastly, we have given a new approach of screening rhizospheric bacteria based on motility on nitrogen deficient media.

Key words: Bacterial Isolates, IMViC, P-solubilization, acid-production, motility

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Introduction

In rhizospheric region of rice plant, roots are closely associated with soil dwelling microbes [1]. Soil microbial populations like Bacillus, Rhizobium, Azotobacter etc bacteria have a lead role in plant anchorage in soil, water and ion absorption, nutrient storage, vegetative growth of plant etc. Plant growth promoting rhizobacteria (PGPR) are soil inhabitants among tremendous amount of micro-organisms in highly complexed soil ecosystem which are able for plant root colonization, plant growth stimulation and crop yield increment [2]. PGPR has a lead role on enhancement of plant growth by various mechanism like atmospheric nitrogen fixation, siderophores production, solubilzation and make available of minerals such as phosphorous, enhanced of uptake of nutrients like nitrogen, phosphorous, potassium, synthesis of phytohormones, indole acetic acid, and gibberlic acid and antifungal activity. PGPR are beneficial for plant and free-living soil bacteria[3]. PGPR are diverse and complex in biosphere. A group of beneficial free-living soil bacteria of PGPR are important for sustainable agriculture and environment. Root pathogenicity may be suppressed by these types of bacteria. Root colonization of the plant occurred by inoculation of these bacteria and can act as biofertilizers and or biopestisides or simultaneously both.

PGPR has a great role in maintaining soil fertility by solubilisation of inorganic phosphorous by phosphate solubilising mechanism which involves organic acid production accompanied by acidification of the medium[4]. Organic acids convert insoluble tricalcium phosphate to di and mono

basic phosphates which enhanced availability of phosphorous to plants. Type, amount and activity of organic acids production depends on ability of different microorganism. Among the organic acids production by soil rhizosphere microbes Gluconic acid and 2-ketoglutonic acid seems to be most important for mineral phosphate solubilisation. Beside these, other organic acids such as acetic acid, citric acid, lactic acid, propionic acid, glycolic acid, oxalic acid, manolic acid, succinic acid, fumaric acid are also impotant for phosphate solubilisation [5].PGPR produced Indole-3-acetic acid (IAA) which stimulates shoot elongation and/or in particular root structures, which could result in more efficient elemental nutrient acquisition by host plants, leading to higher plant growth and development. Organic acids produced by soil microbes involves in mobilization of unavailable phosphorous in soil. There are a number of organic acids such as lactic, citric, 2-ketogluconic, malic, oxalic, malonic, tartaric, and succinic have chelating property which helps phosphorous mobilization and become available to plants[6]. It evidences that Phosphorous uptake by plants increases with increase of organic acids.

Materials and methods

Isolation and purification

Soil samples were collected from surface soil (0-30cm depth) of rice rhizosphere at flowering stage of different agro-ecological zones of North Bengal and then these were stored at 4°C. For isolation of soil microbes, serial dilution of soil sample has been prepared by taking 1.0g soil sample into 9.0ml of sterile double distilled water. Assuming low population of rhizospheric isolates in soil serial dilution up to 3 times dilution has been made. Jensen's Media[7] has used for isolation and purification of isolates. Diluted soil sample has used for spread plate method and these were incubated at 28±2°C. Rhizospheric isolates were detected after 48-72 hours incubation by their colony morphology. These isolates have maintained by preserving it in refrigerator (-20^{0}C) Glycerol stock solutions in the laboratory for various microbiological studies[8].

Methyl red test

MR-VP was dispensed into tubes and sterilized by steaming in Autoclave at 121°C temperature and 15psi pressure for 15 minutes[9]. After inoculation tubes were incubated at 28°C for 3days . Formation of bright red colour was indicator of positive result after adding 5-6 drops of methyl red reagent was added to each tube.

Composition of MR-VP broth is Proteose Peptone, 5.0g; Glucose, 5.0g; NaCl, 5.0g; Distilled water, 1000ml; Ph,7.0 and composition of Methyl red reagent is 0.1g methyl red was dissolved in 300 ml of 95% ethanol and volume was made with distilled water up to 500 ml.

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VogesProskauer test

MR-VP broth was inoculated with bacterial isolates and incubated at 30°C for 48 hrs. After incubation, 0.6 ml of 5% α-napthol (in 95% alcohol) and 0.2ml 0f 40% aqueous solution of KOH were added to 1 ml of culture broth and shaken well[10].

Indole test

Filter sterilized tryptophan was added with nutrient broth and then tryptophan broth were taken into sterilized test tubes. After inoculation all broth were incubated at 28-30°C for 48 hours. Kovac's reagent were added @ 0.5ml into each tubes and then presence of pink colour ring indicate positive results[11].

Utilization of citrate test

Simmon's citrate broth [12] was prepared by adding $MgSO_4.7\ H_2O$, 0.2; $(NH_4)H_2PO_4$, 1.0g; K_2HPO_4, 1.0g; NaCl, 5.0g; Na citrate, 2.0g; Bromothymol blue, 0.08g; Distilled water, 1000ml; Ph, 6.8-7.0 and dispensed into tubes, autoclaved at 121°C for 15 min. After inoculation all broth were incubated at 28°C for 48 hours. Results were recorded after observing the growth and alkaline reaction.

Determination of Phosphorous solubilisation

The quantitative analysis of phosphate solubilisation ability of rhizosphere isolates was measured in vitro condition by determining available soluble phosphate in the Pikovskaya's broth supplemented with 0.5% tri calcium phosphate[13]. The broth medium was inoculated in triplicate by rhizospheric isolates and incubated for 21 days at 28-30°C on rotary shaker at 180 rpm. Available soluble phpsphorous was determined through Phosphomolybdate method in UV- Vis spectrophotometer [14]of supernatant which was isolated by centrifuged at 10,000rpm for 10 min.

Determination of N2 fixing capacity

Rhizospheric isolates were inoculated into 25 ml Jensen's broth media in conical flask and then incubated at 28°C For 21 days with periodic shaking. N fixed was estimated by Kjeldahl method of digestion with Bremmer'sapparatus[15]. Fixation of atmospheric nitrogen of three replication of each isolates was calculated by subtracting the total nitrogen of control flask from that of inoculated flasks. Results were expressed in mg N_2 fixed per g of sugar consumed with respect of sugar present in this medium.

Bacterial motility test

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Three types of bacterial motility (swimming, swarming and twitching motility) test was conducted in Soft Agar method[16] two types of media- (a)nitrogen free media- Jensen's media and (b) nitrogen containing media- Nutrient agar media with different agar concentration. Swimming, swarming and twitching motility was tested in petriplates in these mediums with adding 0.3%, 0.5% and 1.0% agar respectively and incubated at 28-30°C for 96 hours and average diameter of motile bacterial colony was recorded in 24 hour intervals.

Results

Correlation between phosphate solubilization and IMViC test

Biochemical test like IMViC test is one of the assays used to understand the phylogenetic taxa of microorganisms using 'Bergese Manual of Systematic Bacteriology'[17]. We subjected to the soil isolates to the IMViC test and correlated it with the phosphate solubilization properties of the microbes. It was found that the isolates were having variable correlation in Pearson correlation test with the P-solubilising potential. Indole test using Kovac's reagent was having a negative correlation(r= -0.67) with the P solubilization. However, methyl red test (for acid production) was found to have a significant and positive correlation(r= 0.83) with P-solubilization activity (R^2=0.697). The Vogues Proskeur test for acetoin production (r= -0.086) and citrate utilization activity (r= 0.11) was not found to have any correlation with P-solubilisation activity. Based on the results, the scatter plot of methyl red assay and P-solubilization was divided into 4 distinct on overlapping cluster. The Clusters were labelled as 1-4. Cluster 1 included the organisms with high P-solubilization activity and high methyl red absorbance. Cluster 2 included a group of bacteria which were having moderate to high P-solubilizing activity but moderate level ofacid production. Cluster 3 included a group with moderate P-solubilizaton and very less acid production. Last cluster (Cluster 4) included, very low P-solubilization with no acid production. This indicated that methyl red test can act as an indicator of P-solubilising PGPR like properties. This is evident because P-solubilization depends upon the organic acid secreted by bacteria in the soil[18].Indole test remains positive for the organisms which can cleave tryptophan because of the presence of an enzyme called tryptophanase[19]. Tryptophan is a precursor of indole acetic acid thus degradation of tryptophan can lead to lower amount of acid production which in turn can minimize the P-solubilization. This explains the negative correlation with the Indole test by Kovac's reagent. Vogues Prausker (VP) test is for the conversion of organic acid to acetoin. Thus, organisms which were negative for methyl red test should have been positive for VP test. However, this condition was not satisfied. This signifies that the amount of acid produced

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need not to be important but the type of acid produced is also equally important.

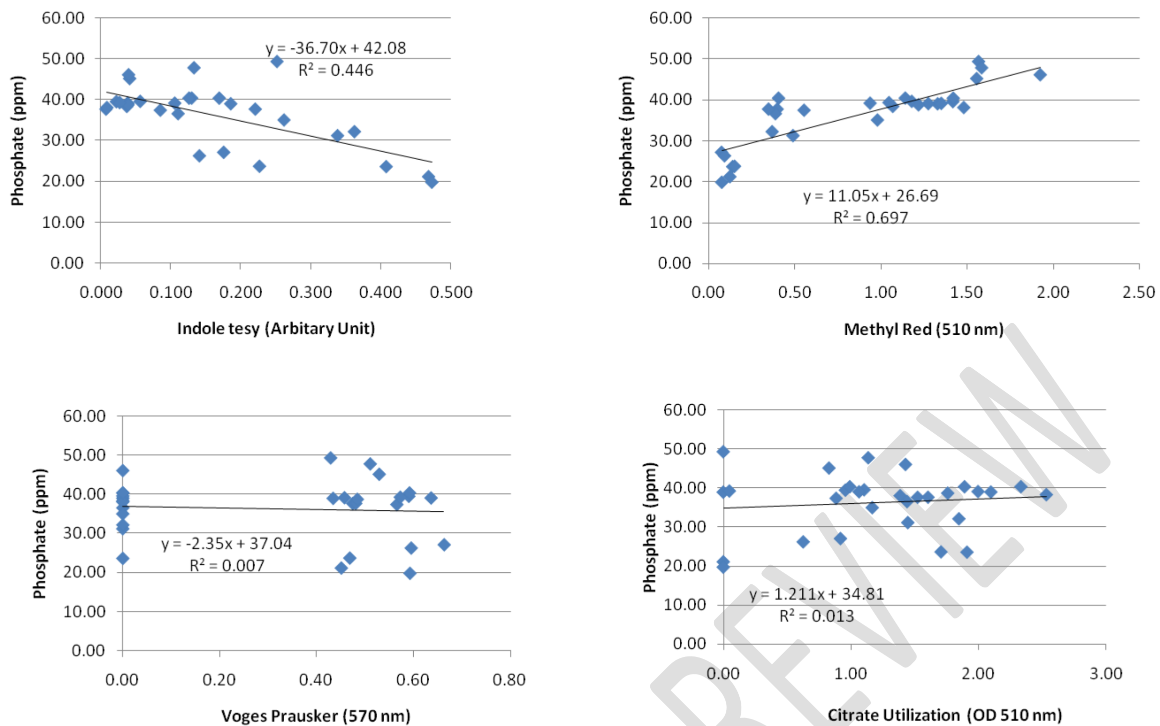

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Figure 1: Correlation between IMViC parameters and P-solubilization activity. Correlation between P-solubilization and Indole test (Top left), methyl red test (top right), VogeusProskeur test (Bottom left) and citrate utilization test (bottom right).

MR test-based clustering of bacterial strains against their P-solubilization properties

Among the strains used for the study, AZ-037, 079, 173, D-008 and M-163 were able to solubilize >40ppm of phosphate over 21 days. These organisms were having a good amount of acid production based on the colorimetric absorbance of methyl red (Cluster 1). This correlates with the fact that acid production is a prerequisite for P-solubilization. The organisms belonging to the second cluster had moderate to high P solubilization activity (between 30 to 40 ppm) with moderate acid production. The cluster 4 had less than 30ppm of P-solubilization activity with no acid production, thus signifying a cluster deficient in this PGPR activity. Interestingly, cluster 3 has a low acid production but moderate P solubilization, signifying a unique property of this group of bacteria (namely AZ-153, 156, 183, D-019, M-071, 077, 139) which can solubilize phosphate with low acid. This reflects the heterogeneity of the microbial metabolism for production of acids. This also hinted towards the production of a weak acid which is somehow more potent in solubilizing the inorganic phosphate. Further studies will be required for understanding the mechanism. This also hinted towards the possibility of specificity of certain organic acids towards P-solubilization. Thus, we can conclude that the diversity of P solubilizing organisms cannot be determined based on only the acid production

alone. Supporting information regarding the type of acid can help in screening some good PGPRs for the usage of P- solubilization.

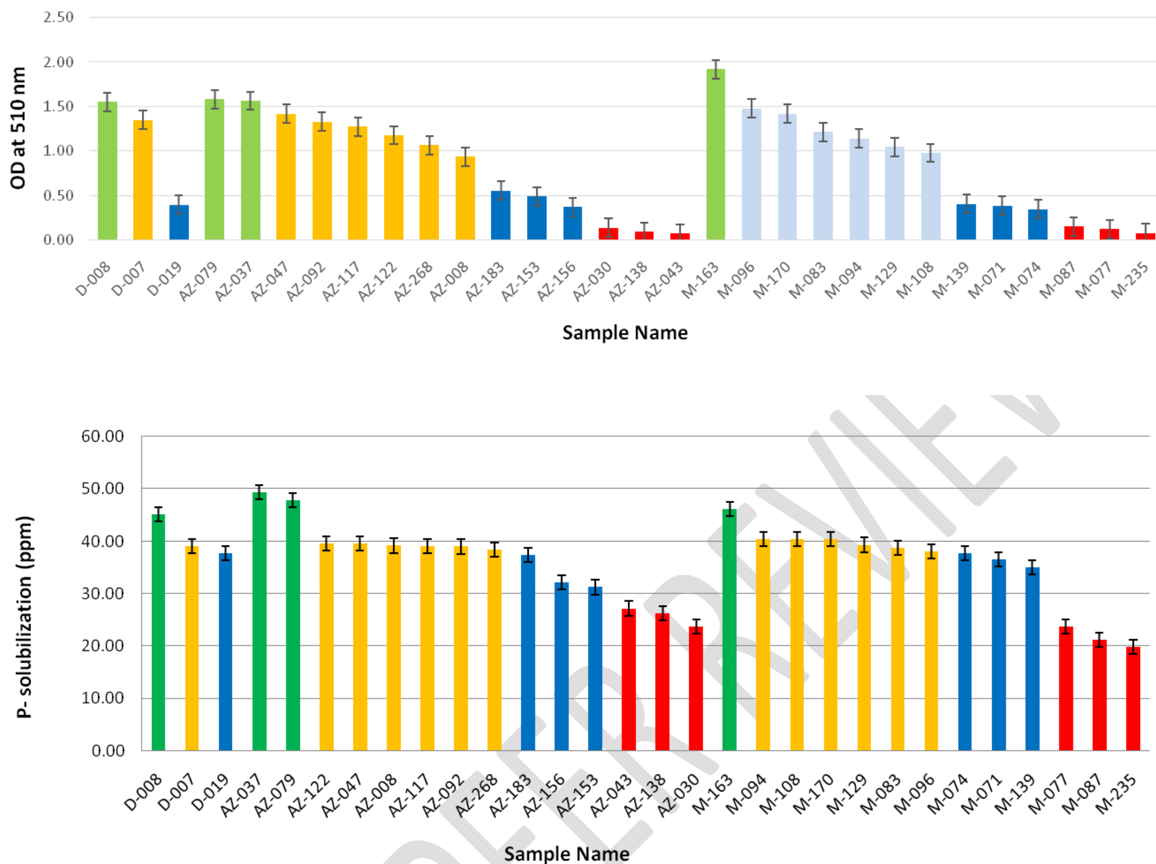

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Figure 2: Methyl red absorbance (top) and P solubilization (bottom) activity of the bacterial isolates collected from various region of north Bengal. The coloured bar indicated the cluster to which the bacteria belongs. Green bar (Cluster 1), yellow bar (cluster 2), blue bar (cluster 3) and red bar (cluster 4). The solid bar and error bar represent mean and standard deviation of 3 replicates.

Characteristics in nitrogen deficient condition

Since the bacteria were isolated in nitrogen deficient media, we analysed the total nitrogen assimilated by the bacteria. The total nitrogen pool of the media was assessed. Among the isolates, AZ-037, 117,122,138,268, M-077, 083 were found to have incorporated a very high amount of nitrogen (Mean value >300ppm). Probably these organisms are nitrogen fixers. However, the isolate D-008 was found to have very low assimilation (mean <200ppm). This organism can be considered as a nitrogen scavenger. Other isolates were having nitrogen content in the range of 200-300 ppm. This intermediate group cannot be classified as nitrogen fixer or scavenger. More detailed screening will be required to classify these group of organisms.

Since nitrogen fixation is an energy exhaustive process [20], we analysed the swimming motility of every isolate in the nitrogen deficient media. Motility by requires a large amount of energy [21]. We

analysed swimming motility of these organisms in nitrogen deficient media with 0.3% agar. The spotted colony was monitored for 96 hours. To our surprize, we got 4 distinct class of bacteria were observed. These are reported as follows:

a. A hyper motile strain (M-71) signifying that this organism can channelize its energy for motility even under nitrogen deficient condition. This bacterium can be used as potential biofertilizer strain as it can migrate very fast under nitrogen deficient condition. One can speculate that this bacterium can migrate and colonize the soil with limited nitrogen source and can act as simultaneously act as nitrogen source.
b. High motility strains: M-87, 139, 163, 170, 235, D-008,019, AZ-008,153, 268, AZ-ICAR. These bacterial isolates are able to migrate fast even under nitrogen deficient condition signifying these can colonize in agricultural fields with nitrogen deficiency. This group can be used as potential biofertilizer strains because they can also channelize their residual energy for motility under nitrogen deficient condition.
c. Moderate motility: AZ-117, 173, 037, 092, 043, 047, 138, 030, 156, 079, D-007, M-129, 77, 83, 189, 108, 74. This group cannot migrate or colonise faster in nitrogen deficient condition signifying the motility is highly dependent on nitrogen. These bacteria cannot channelize the residual energy for migration. They also share some P-solubilizers but because of low motility this group can be used only as seed priming for field application.
d. The last group is of non-motile bacteria (AZ-122, 183, M-94, 96) which could not migrate in media deficient of nitrogen. These strains either lack flagella or could not synthesize flagella due to nitrogen limitation. This group cannot be considered effective for field application as they can not survive because they cannot migrate for finding new reserve of nutrient in soil. Thus, the only possible role of this group can be in well fertilized field where they do not suffer from nutrient deficiency.

In conclusion, we can postulate that these screening methods can help in detecting fast migrating bacterial population which does not depend on nitrogen concentration in soil thus providing a more effective way of screening of potential isolates for biofertilizer production. In future, these techniques can be complemented with other biochemical parameters for assessing more PGPR properties. This kind of an analysis can be useful for classifying PGPRs based on biochemical tests.

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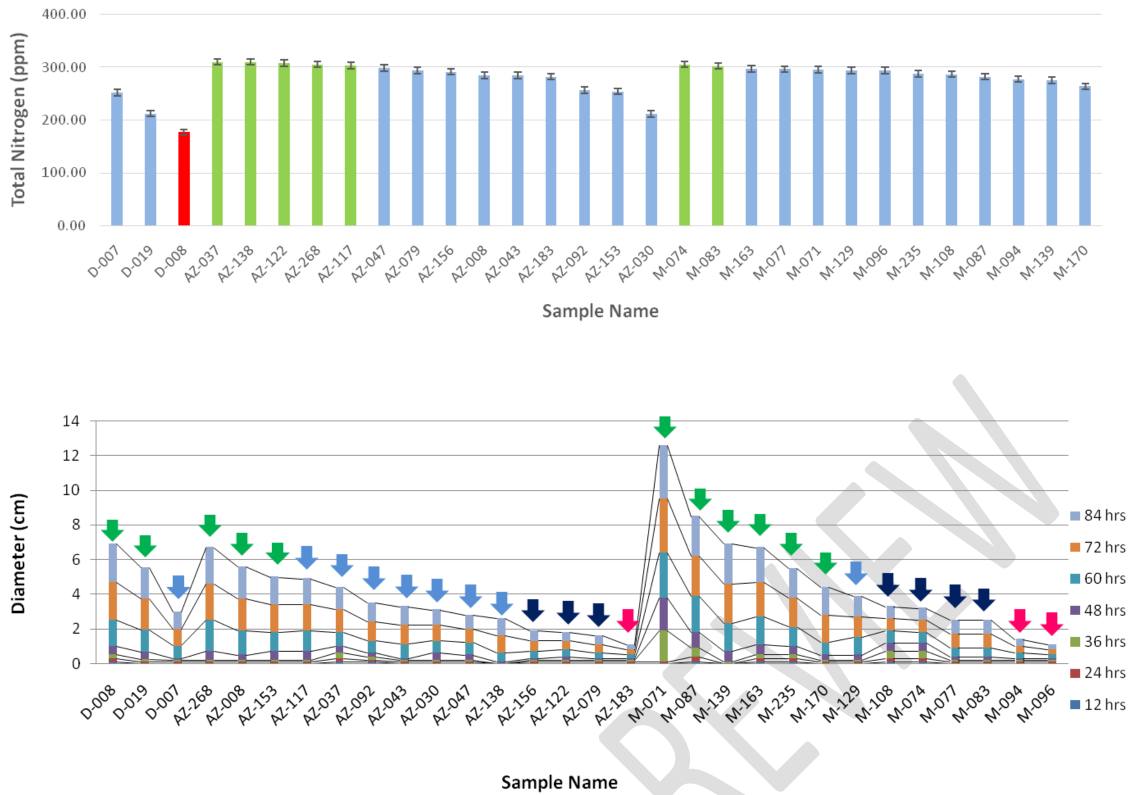

Figure 3: Comparison of nitrogen assimilation and migration in nitrogen deficient growth media. (A) Total nitrogen content in the media after bacterial growth for 21 days. Green bar, blue bar and red bar represents high (>300 ppm), medium (200-300 ppm) and low (<200 ppm) nitrogen respectively.The solid bar and error bar represent mean and standard deviation of 3 replicates. (B) Colony spread in swimming motility assay in 0.3% agar and nitrogen deficiency across different time. Green arrow represent highpermotility, sky blue arrow represent high motility, blue arrow represent moderatemotility and red arrow represent less or non motile bacteria.

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Discussion And Conclusion

It has previously been shown that higher organic acid producing bacteria solubilise more amount of phosphorous from insoluble TCP[22]. Organic acids act as a regulator of the change of microenvironment (reduce pH) also change the chemical composition of insoluble phosphorous sources by formation of weak ligand, chelation of metal and liberation of phosphorous in solution[23] . Amount of solubilised phosphorous is also positively correlated with the quantity of produced organic acids by phosphate solubilising microorganisms. Phosphorous solubilisation and tryptophanase enzyme production by the organisms are negatively correlated. Acetoin content and citrate utilization have not any effect on phosphorous solubilisation. Acetoin form

acetylmethylcarbinol by fermentation of glucose in alkali media. Citrate take part in microbial metabolic process and form pyruvate in alkaline condition [24].

Based on N2 fixing capacity, rhizospheric isolates are grown in mitrogen free media and they are classified into three groups based on nitrogen fixing in media. Seven isolates are Nitrogen fixer (above 300 ppm nitrogen), most of the isolates are in intermediate position of fixer and scavenger (200-300ppm nitrogen) and only one isolate is identified as nitrogen scavenger (less than 200ppm nitrogen). In motility test we found that M-71 strain is highper motile in nitrogen deficient condition and M-87, 139, 163, 170, 235, D-008,019, AZ-008,153, 268, AZ-ICAR are high motile isolates. These two groups may be used for making potential biofertilizer. Moderately motile isolates like- AZ-117, 173, 037, 092, 043, 047, 138, 030, 156, 079, D-007, M-129, 77, 83, 189, 108, 74 cannot migrate faster in nitrogen deficient condition and their motility depends on the presence of nitrogen. These are not usable as biofertilizer but these strain may be used as seed priming agent based on their phosphorous solubilising capacity. The non-motile group bacteria like AZ-122, 183, M-94, 96 which could not migrate in nitrogen deficient media because either lack flagella or could not synthesize flagella due to nitrogen limitation. This group cannot be considered effective for field application as they cannot survive.

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