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ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBLIZING BACTERIA FROM ANAND AGRICULTURE SOIL.

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ABSTRACT

Phosphorus is vital to seed formation and its content is higher in seeds than in any other part of the plant. It helps plants to survive winter rigors and also contributes to disease resistance in some plants. Also known to improve quality of many fruits, vegetables and grain crops.The inoculation of P-solubilizing microorganisms is a promising technique because it can increase P availability in soils fertilized with rock phosphates. In the present work we have made efforts to isolate the phosphate solubilizing microorganism form soil followed by its biochemical and molecular characterization

Key words: Phorphorus, PVK media, halo zone, 16S rRNA amplification

INTRODUCTION

Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant. An adequate supply of phosphorus in the early stages of plant growth promotes physiological functions including early root formation, and is important for laying down the primordia for reproductive parts of plants. It is vital to seed formation and its content is higher in seeds than in any other part of the plant. It helps plants to survive winter rigors and also contributes to disease resistance in some plants. Also known to improve quality of many fruits, vegetables and grain crops. Biological Nitrogen Fixation depends appreciably on the available forms of phosphorus. Phosphorus (P) is an important structural constituent of nucleic acids, phytin and phospholipids. Mineral forms of phosphorus constitute the biggest reservoirs of phosphorus, represented primarily by rocks and deposits formed during geological age. The principal characteristic of these primary minerals (oxyapatite, hydroxyapatite, apatite) is their insolubility. A large portion of soluble inorganic phosphate applied to agricultural soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dadarwal*et al*., 1997).

PSMs include different groups of microorganisms, which not only assimilate phosphorus from insoluble forms of phosphates, but they also cause a large portion of soluble phosphates to be released in quantities in excess of their requirements.Species of *Aspergillus*and *Penicillium*are among fungal isolates identified to have phosphate solubilizing capabilities. Among the bacterial genera with this capability are *Pseudomonas, Azospirillum, Bacillus, Rhizobium, Burkholderia, Arthrobacter, Alcaligenes, Serratia, Enterobacter, Acinetobacter, Flavobacterium and Erwinia*(Rodriguez *et al*., 1996). Seed or soil inoculation with PSMs is known to improve

solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields (Jones et al., 1994) PSMs are a low-cost solution that enriches the soil giving a thrust to economic development without disturbing ecological balance.The inoculation of P-solubilizing microorganisms is a promising technique because it can increase P availability in soils fertilized with rock phosphates [Reyes et.al(2002)]. Several authors reported yield increasing on wheat [Whitelaw *et a*l (1997)], onion [Vassilev *et. al* (1997)], alfalfa [Rodríguez 1999] and soybean [Abd-Alla *et. al* (2001)] through simple inoculation of P-solubilizing fungi (PSF). Inoculation of phosphate solubilizing fungi and mycorrhizal fungi improves the physio-chemical, biochemical and biological properties of rock-P amended soil [Caravaca, F *et .al* (2004)].

MATERIALS AND METHODS

Sample collection

Total of 8 samples were collected from different ecological sources in sterilized bottles. Ecological sources included were mainly agriculture land.

Isolation of phosphate solubilizing Bacteria from soil sample

Preparation of media for isolation of PSM

Readymade formulation of dehydrated media from the makers of 'HI MEDIA' was used for preparing solid as well as liquid media. 9 cm diameter petri plates were used for routine culture of bacteria.

Preparation of inoculums from soil sample and inoculation

Acidic soil sample from nearby village of Anand, (Gujarat) was collected and dissolved in sterile distilled water and then serially diluted up to 10^{-7}.Then after 100µl of 10^{-7} serially diluted sample spread on PVK agar and the plates were incubated at 37^{o}C for 24 hrs. After incubation the bacterial colonies having clear zone of phosphate solubilization were selected and streaked on new PVK agar plates to get isolated pure colonies. Further purification was carried out on the same media.

Microscopic study of Bacteria

Size, shape, arrangement and gram's nature of the isolates were studied. For grams staining smear was prepared from the isolated culture on clean grease free microscopic glass slide, heat fixed and stained. The stained smear was observed under microscope (oil immersion lance-100X).

Identification of Bacterial isolates through Biochemical tests.

Biochemical test were performed as suggested by Garrityet et al., 2004, which include following tests Gram staining, IMVIC reaction, Catalase test, Starch hydrolysis test, Oxidation fermentation test, Phenyl alanine deamination test, Lead acetate paper strip test, Ammonia production test, Nitrate reduction test, Gelatin hydrolysis test, Urea hydrolysis test, Dehydrogenase test, Casein hydrolysis test, Citrate utilization test, Indole production test, Triple sugar iron (TSI) test, Carbohydrate fermentation test (Glucose, Fructose, Xylose, and Sucrose), Motility test, Endospore staining and Capsule staining.

Molecular characterization of Bacterial isolates

Isolation of genomic DNA

Pellet of bacterial cell was suspended in 2 ml Tris-EDTA (pH 8.0), 250 µl 10% SDS, 50 µl Lysozyme (20 mg/ml), and incubated at 37^{o}C for 1 hr. then 500µl 5M NaCl and 100µl CTAB were added and incubated for 10 min at 65^{o}C. Then phenol, chloroform and isoamyl alcohol were added and centrifuged for 10 minute. Supernatant was extracted with chloroform:isoamyl alcohol and finally precipitated with 1/10 volume of 7.5 M Ammonium acetate (pH-7) and double the volume of chilled ethanol. Pallet was suspended in 30 ml of sterile water.

Amplification and automated fluorescent nucleotide sequencing of the 16S ribosomal DNA fragment

Bacterial ribosomal gene analysis was performed as described by Weisberg et al. (1991), with minor modifications. The PCR products were purified by using a nucleotide removal kit (Qiagen, Germany)

and the sequencing products were purified according to the anufacturer's protocol. Base calling was performed with Applied Biosystems Sequence Analysis Software (version 5.2) and quality value bar above 40 was considered to be acceptable for further analysis DNA sequence similarity search was performed using the BLAST algorithm (Altschul et al. 1994, 1997).

16srRNA PCR of isolated bacterial DNA

100 ng of the genomic DNA was used for performing 16s PCR and the composition of reaction mixture is a below

Components	Quantity (µl)
2 X PCR Master Mix	15.0
Forward Primer (10pmole/µl)	1.0
Reverse Primer(10pmole/µl)	1.0
Nuclease Free Water	9.0
DNA Template	4.0
Grand Total	30.0

Primers and (forward reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
16S rRNA	94oC 3min	94oC 40sec	55oC 45sec	72oC 75sec	72oC 3min

Agarose Gel Electrophoresis

To confirm the targeted PCR amplification, 8µl of PCR product from each tube was mixed with 2µl of 6X gel loading buffer from each tube and electrophoresed on 1.0 % agarose gel along with 100 bp DNA Ladder (Bangalore genie) and stained with ethidium bromide at constant 80 V for 45 minutes in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (Alpha imager, Alpha innotech). .

ARDRA

For ARDRA 16S rDNA was amplified and restricted digested by the enzyme ECO-RV. Steps involved in this method are amplification of 16S r-DNA was performed using 30ng DNA in 25µL of total volume reaction. A 10µL aliquot of each PCR reaction was incubated with 1-2 units of one restriction enzyme and the mixture was incubated at 370C for 3 hours. The restriction products were then analyzed by electrophoresis on 1.5% agarose in 1X-TBE buffer at 100 V and the gel was visualized in gel documentation system (Alpha Innotech).

SDS-PAGE:-

Gel preparation and sample preparation:

A 10% resolving gel and a 5% stacking gel was formed in the Biorad SDS –PAGE unit. 2 X SDS-PAGE sample buffer was added to the loosened pellet, the protein lysate so obtained was centrifuged at 12,000 rpm for 12 minutes, supernatant was collected in a new vial and heated at 950C for two minutes followed by cooling of the samples at room temperature and giving a short spin, the samples thus prepared were then loaded in the gel along with a mid-range protein marker of 14KDa-95KDa (SRL BIOLITTM).The samples were allowed to run at 100 V for 5 hours followed by overnight staining of the gel in 1% Commassie

Brilliant Blue. The destaining of gel was carried out the next day and the bands were visualized and documented by gel documentation system (Alpha imager, Alpha innotech).

RESULT

Screening of Ecological Sources:

Location of soil sample collection was chosen because of the possibility of occurrence of phosphate solubilizing microbes. Sampling was done at various sites, in order to maintain uniform representation of the micro flora in and around the collection area. For initial growth of micro flora PVK medium was used embedded with tricalcium phosphate as phosphate source. Preliminary investigation of these cultures in PVK media embedded with tricalcium together with agitation and aeration for 5 day allowed microbial solubilization of P with fall in pH. This was followed by the dilution plating in order to isolate the single colonies. The concentration of 1.5% agar adequately maintained the desired texture of the solid medium, while simultaneously retained enough moisture to promote microbial growth.

Its been reported that organisms capable of doing phosphate solubilization give clear zone around the colony by which it can be concluded that they are phosphate solubilizing microorganisms. Five colonies gave clear zone by which it was confer that they can solubilize phosphate. **Fig 1.**

Sr.No	Biochemical Test	AB- 01	AB-O2	AB-03	AB-04	AB-05
1.	Grams Staining	+	-	+	+	-
2.	Capsule staining	+	-	+	+	-
3.	Motility	-	-	-	+	-
4.	Methyl Read Test	+	-	+	+	-
5.	VogesProskau (VP) Test	+	-	+	+	-
6.	Catalase Test	-	+	-	-	+
7.	Starch Hydrolysis Test	-	-	-	+	-
8.	Oxidation Fermentation Test	+ +	+ -	- -	_	+
9.	Phenylalanine deamination test	+	-	-	+	+
10.	Lead acetate paper strip test	-	-	+	+	-
11.	Ammonium production test	+	-	+	-	+
12.	Nitrate reduction test	+	+	+	-	+
13.	Gelatin hydrolysis test	-	+	-	-	-
14.	Urea hydrolysis test	-	+	-	-	-
15.	Dehydrogenase test	+	-	-	-	+
16.	Casein hydrolysis test	-	-	-	+	+
17.	Citrate utilization test	+	-	+	-	+
18.	Indole production test	-	+	-	-	+
19.	TSI test(Triple sugar test)	+	-	-	+	+
20.	Carbohydrate fermentation test Glucose Fructose Xylose Sucrose	 + + -	 + + -	 + + +	 + + +	 + + -

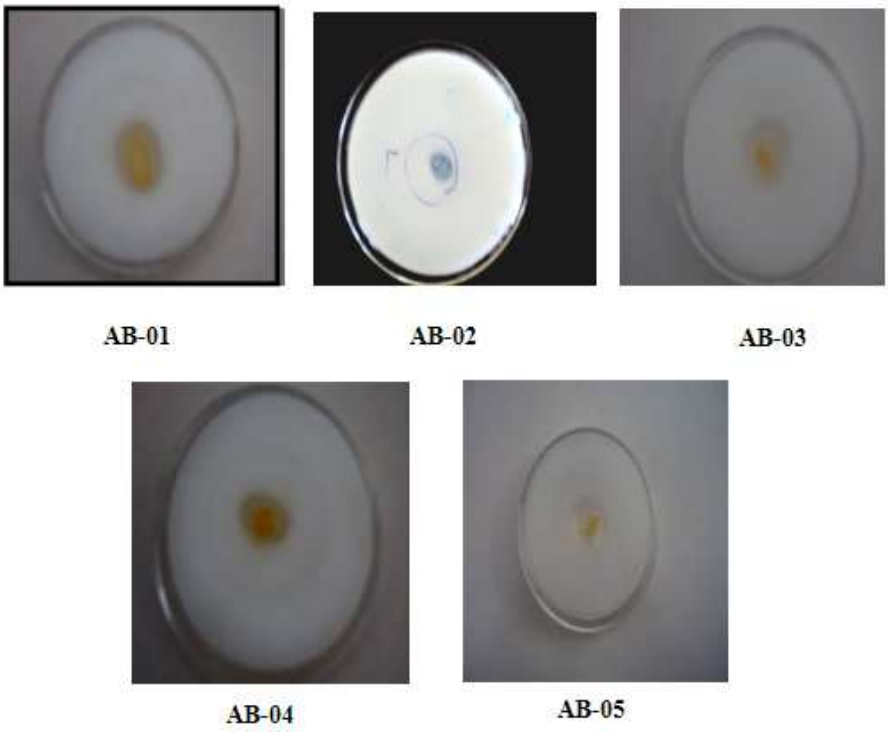


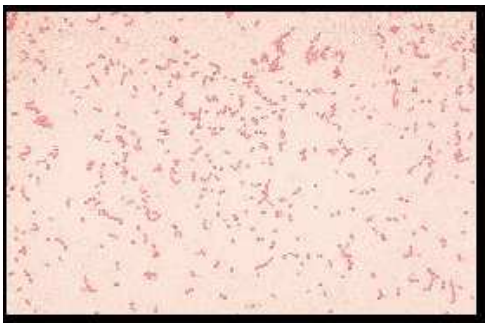
Figure 1: ***Halo Zone around the colony on PVK media confirm Phosphate solubilizing bacteria***

Identification of Bacterial isolates through Biochemical tests:
All the strain was subjected to the biochemical test for their identification. The results are summarized in **Table 2.**

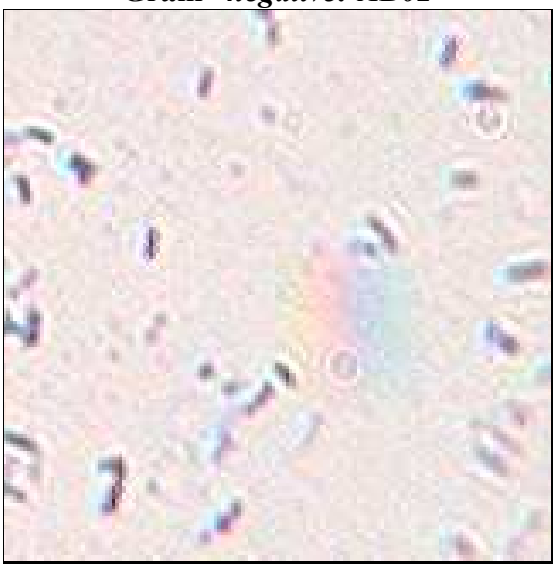
Table- ***Colony characteristics of the individual isolates***

Table- ***Biochemical characterization of the isolates***

Test	**AB-01**	**AB-02**	**AB-03**	**AB-04**	**AB05**
Size	Small	Large	Big	Small	Large
Shape	Round	Round	Round	Punctiform	Round
Margin	Entire	Entire	Entire	Entire	Entire
Elevation	Raised	Flat	Raised	Flat	Flat
Surface texture	Smooth	Smooth	Smooth	Smooth	Contoured
Consistency	Moist/Butyrous	Moist/ Butyrous	Moist/ Butyrous	Moist/ Butyrous	Viscous
Optical Character	Translucent	Translucent	Opaque	Translucent	Translucent
Pigmentation	Orangish	White	Yellowish	Yellowish	Yellowish

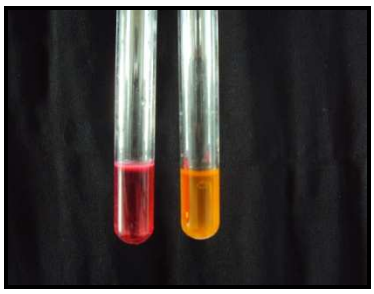


Gram –*negative:-AB02*



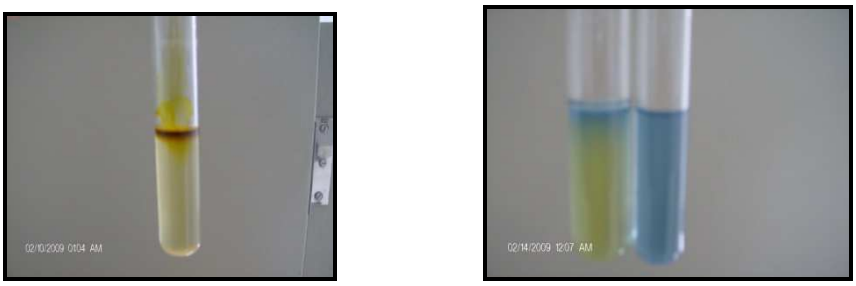
Gram-*positive:-AB01*

Figure-8:- *Gram staining of culture*



Positive glucose fermentation Positive citrate utilization

Casein hydrolysis test



Positive indole testPositive dehydrogenase production test

Figure-9:- ***Some of the positive Biochemical Tests.***

Isolation of genomic DNA and Estimation

DNA of all the strain was isolated and was visualized by running it on 1.0% (w/v) agarose gel. The gel was visualized in UV transilluminator and photographed subsequently. Isolated DNA, after organic extraction, appeared a thick high molecular weight band with a faint back ground. Optical density was taken at 260/280 nm. All the DNA were found to be pure.

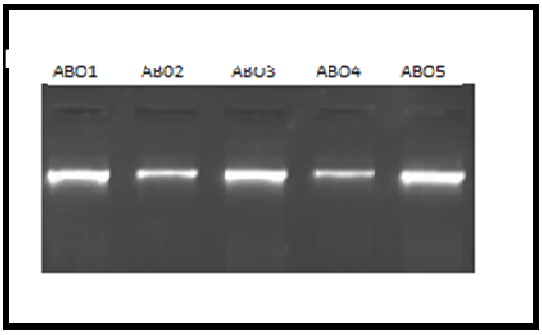


Figure 10: ***DNA of AB-01, AB-02,AB-03,AB04 and AB05***

16s rRNA PCR of isolated bacterial DNA and Sequencing

All the strain gave 1.5 Kb band upon amplifying with 16s rRNA universal primer. Upon sequencing AB02 is *Burkholderia cenocepacia* and AB04 can be a *Bacillus sp*.

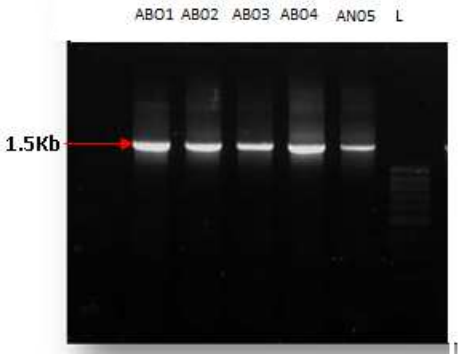


Figure 11: ***16s RNA PCR***

Amplified r-DNA Restriction Analysis (ARDRA):

16S rPCR product was digested with Eco RV in order to see the polymorphism. Results show that four samples (AN-01, AN-02, AN-03 and AN-05) had no polymorphism while one sample (AN-04) showed different banding pattern. This showed that AN-04 had had Eco RV recognition sit in the 16s rRNA amplified product.

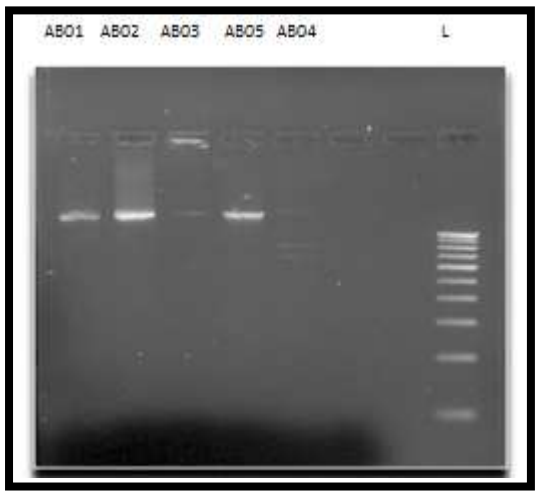


Figure Eco R V digested 16s RNA PCR product

16s RNA sequencing:

16s rRNA PCR products of all isolates were send for sequencing. The sequences obtained were studied using BLAST in NCBI database to find out maximum percentage homology with known bacterial DNA sequences to identify the isolates. It was found that AB-02 has 90% homology with *Burkholderia cenocepacia*. While AB-04 had 82% homology with *Bacillus sp*.

Protein profiling of the Isolates:

Upon performing protein profiling it was found that all the isolates did not gave same banding pattern. There were few bands which were common in isolates but rest showed polymorphism. This results help to interpret that which isolates may be belonging to same group and which are from the divergent group.

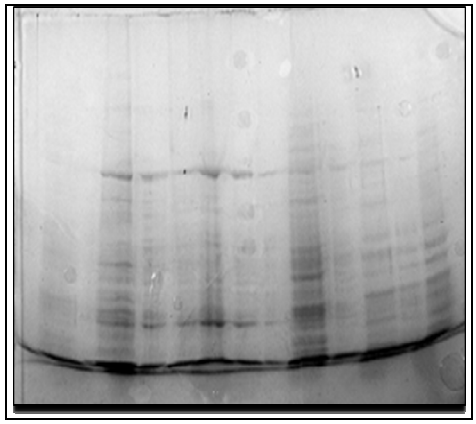


Figure 13: ***SDS-PAGE of all the isolates***

REFERENCES

1. Abad-Allah, M.H . (1994) Phosphates and the utilization of organic phosphorus by Rhizobium leguminosarumbiovarviceae. *Letters of Applied Microbiol.*, 18: 294-296.
2. Abd-Alla, M.H., S.A. Omar and S.A. Omar, (2001) Survival of rhizobia/bradyrhizobia and a rock phosphate- solubilizing fungus *Aspergillusniger*on various carriers from some agro-industrial waste and their effects on nodulation and growth of faba bean and soybean. *Journal of Plant Nutrition*, 24(2): 261-272.
3. Altschul SF, Boguski MS, Gish W, Wootton JC, 1994.Issues in earching molecular sequence databases.Nature Genetics 6:119–129.
4. *Agnihotri* V P, (1970) Solubilization of insoluble phosphate by some soil fungi isolated from nursery seedbeds. Can. J. Micro. 16,877-880.
5. Ahmad Ali Khan 1, GhulamJilani 1,*, Mohammad SaleemAkhtar 1, Syed Muhammad SaqlanNaqvi 2, Mohammad Rasheed.,(2009)Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production
6. Almas ZAIDI, Mohammad Saghir KHAN.,(2005)Co-inoculation Effects of Phosphate Solubilizing Microorganisms and Glomusfasciculatum on Green Gram-Bradyrhizobium Symbiosis
7. Barea, J.M., C. Azcón-Aguilar and R. Azcón, (1997). Interactions between mycorrhizal fungi and rhizosphere microorganisms within the context of sustainable soil-plant systems. *In*: Gange, A.C., Brown, V.K. (*Ed.).*Multitrophic interactions in terrestrial systems. Cambridge: *Blackwell Science*, pp: 65-77.
8. Caravaca, F., M.M. Alguacil, R. Azcón, G. Diaz and A. Roldan,(2004). Comparing the effectiveness of mycorrhizal inoculation and amendment with sugar beet, rock-phosphate and *Aspergillusniger*to enhance field performance of the leguminous shrub *Dorycni*.
9. Cunningham, J. E. and Kuiack, C . (1992), Production of citric acid and oxalic acid and solubilization calcium phosphate by penicilliumbillai.*Appl. Environ. Microbiol.*,58, 1451–1458.
10. Dadarwal , Yadav K.S and , K.R.(1997). Phosphate solubilization and mobilization through soil microorganisms. In: biotechnological approaches in soil microorganisms for sustainable crop production (Dadarwal, K.R., Ed.) pp. 293-308. Scientific Publishers, Jodhpur.
11. Datta, M., S. Banik, and R. K. Gupta. (1982). Studies on the efficacy of a phytohormone producing phosphate solubilizing Bacillus firmus in augmenting paddy yield in acid soils of Nagaland. *Plant soil* 69: 365-383.
12. Fasim F Ahmed N, parsons R, Gadd GM (2002). Solubilization of Zinc salts by bacterium isolated by the air enviourment of tannery FEMS Micro. Lett 213,1-6.
13. Gadd, G.M . (1999). Fungal production of citric and oxalic acid: Importance in metal speciation, physiology and biogeochemical processes. Advances in Microbial Physiology, 4: 47-92 .
14. Glick, B.R., (1995). The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.* 41, 109–117.
15. Goldstein, A.H., (1995). Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by Gram negative bacteria. *Biol. Agric. Hort.* 12, 185–193.
16. Goldstein, A.H., Braverman, K., Osorio, N., (1999). Evidence for mutualism between a plant growing in a phosphate-limited desert environment and a mineral phosphate solubilizing (MPS) bacterium. *FEMS Microbiol. Ecol*. 3, 295–300.
17. Gyaneshwar, P., Naresh K. G. and Parekh, L, J.*Curr. Sci.*, (1998)74, 1097–1099.

18. Halder, A.K., Mishra, A.K., Bhattacharya, P., Chakrabarthy, P.K., (1990). Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. *J. Gen. Appl. Microbiol.* 36, 81–92.
19. Hilda, R., Fraga, R., (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv*. 17, 319–359.
20. H.S. Han1, Supanjani2, K.D. Lee2.,(2006)Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber
21. Illmer P, Schinner F. (1992). Solubilization of hardly-soluble AlPO4 with P-solubilizing microorganisms. *Soil Bio and Biochem*. 24: 389-395.
22. Illmer, P. and F. Schinner, (1992). Solubilization of inorganic phosphate by microorganisms isolated from forest soils. *Soil Biol. Biochem*., 24: 389-395.
23. Iman M. El-Azouni(2008). Effect of Phosphate Solubilizing Fungi on Growth and Nutrient Uptake of Soybean (*Glycine max* L.) *plants Journal of Applied Sciences Research*, 4(6): 592-598.
24. Kim, K.Y., Jordan, D., Krishnan, H.B., (1997) Rahnellaaqualitis, a bacterim isolated from soybean rhizosphere, can solubilize hydroxyapatite. *FEMS Microbiol. Lett.* 153, 273–277.
25. Kpomblekou, K., Tabatabai, M.A., (1994). Effect of organic acids on release of phosphorus from phosphate rocks. *Soil Sci.* 158, 442– 453.
26. Kucey, M. N., Janzen, H. H. and Legett, M. E, (1989), *Adv. Agron.*, 42, 198–228.
27. Motsara, M.R., P.B. Bhattacharyya and B.Srivastava, (1995). Biofertilizers-their description and characteristics In: BiofertilizerTechnology,Markting and Usage, A sourcebook-cum-Glossary.Fertilizer development and consultation organization 204-204 A Bhanot Corner, 1-2 Pamposh Enclave, New Delhi, 110048, India, pp: 9-18.
28. Martin, S.M. & Steel, R. (1955). Effect of phosphate on production of organic acids by Aspergillusniger. Can. J. Microbiol., 1, 470-472.
29. Mattey, M. (1992). The production of organic acids. Rev. Biotechnol., 12, 87-13-50
30. Nahas E. (1996). Factors determining rock phosphate solubilization by microorganism isolated from soil. W.J. MicrobiolBiotechnol. 12:567-72.
31. Narsian, V., Thakkar,J., Patel, H.H., (1995). Mineral phosphate solubilization by *A.aculeatus*. Indian Journal of Experimental Biology 33, 91-93.
32. Narsian,V., Patel, H., (2000). *Aspergillusaculeatus*as a rock phosphate solubilizer. Soil Biology & Biochemistry 32: 559-565 plant available phosphorus. Adv. Agron. 42:199-228.
33. N Pradhan and LB Sukla. (2005). Solubilization of inorganic phosphates byfungi isolated from agriculture soil. *African Journal of Biotechnology*. 5:850-854.
34. Nguyen C, Yan W, Le Tacon F, Lapyire F(1992) Genetic variability of phosphate solubilizing activity by monocaryotiv and dicaryotic mycelia of the ectomycorrhizal fungus laccaria bicolor (Maire) P D Orton, Plant Soil 143,193-199.
35. Omar, S.A.The.(1998) role of rock-phosphatesolubilizing fungi and vesicular-arbuscularmycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. World *Journal of Microbiology and Biotechnology*, 14: 211-218.
36. Rajan SSS, Watkinson JH, Sinclair AG., (1996). Phosphate rocks for direct application to soils. Adv. Agron. 57: 77-15.
37. Reddy, M., Kumar, S., Khosla, B., (2002). Biosolubilization of poorly soluble rock phosphates by *Aspergillustubingensis*and *Aspergillusniger*. Bioresource Technology 84:187-189
38. Reyes I, Bernier L, Simard R, Tanguay PH, Antoun H(1999). Characterestics of phosphate solubilizationbyu an isolate of a tropical Penicilliumregulosom and two UV induced mutants. FEMS MicrobiolEcol 1999; 28:291-5
39. Reyes, I., L. Bernier and H. Antoun, (2002). Rock phosphate solubilization and

colonization of maize rhizosphere by wild and genetically modified strains of Penicilliumrugulosum. *Microbial Ecology*, 44: 39-48.

40. Rodríguez, R., N. Vassilev and R. Azcón, (1999.). Increases in growth and nutrient uptake of alfalfa grown in soil amended with microbially-treated sugar beet waste. *Applied Soil Ecology*, 11: 9-15.
41. SadafShahab and Nuzhat Ahmed, (2008). Effect of various parameters on the efficiency of zinc phosphate solubilization by indigenous bacterial isolates *African Journal of Biotechnology* Vol. 7 (10), pp. 1543-1549, 16 May.
42. Seshadri,S.,S. Ignacimuthu,C.Lakshminarasimhan, (2004). Effect of nitrogen and carbon sources on the inorganic phosphate solubilization by different Aspergillusnigerstrains. *Chemical Engineering Communications,* 191: 043-1052.
43. Scheffer F, Schachtschabel P. Lehrbuch der Bodenkunde. Stuttgart: Enke, (1998).The role of rock *phosphate solubilizing* fungi and vesicular arbuscularmycorrhiza (VAM)
44. Surange, S., Wollum, A.G. II, Nikhil Kumar and Nautiyal, C.S., (1997).Characterization of *Rhizobium* from root nodules of leguminous trees growing in alkaline soils. Can. J. Microbiol. 43, 891-894.
45. Sun Chul Kang, ChulGyuHa,TaeGeun Lee and D. K. MaheshwariSolubilization of insoluble inorganic phosphates by a soil-inhabiting fungus Fomitopsis sp. PS102.
46. Tiwari V.N. Lehri L.K.,Pathak A.N.(1989) Effect of inoculating crop phospho-microbes. Expel. Agric. 25: 47-50.
47. Vassilev, N., M. Toro, M. Vassileva, R. Azcón and J.M. Barea, (1997). Rock phosphate solubilization by immobilized cells of *Enterobacter* sp. in fermentation and soil conditions. *Bioresource Technology*, 61: 29-32.
48. Varsha NHH (2002) Aspergiliusaculeatus as a rock phosphate solubilizer. Soil. Biol. Biochem. 32..599-565.b(2004.) Phosphate solubilization by *Penicillium*spp. closely associated with wheat roots. *Biology and Fertility of Soils*, 40: 36-43.
49. Whitelaw, M.A., T.J. Harden and G.L. Bender, .(1997) Plant growth promotion of wheat inoculated with *Penicilliumradicum*sp. nov.*Australian Journal of Soil Research*, 35: 291-300.
50. Weisburg WG, Barns SM, Pelletier DA, Lane DJ, 1991.16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703
51. Whitelaw, M.A., T.J. Harden and G.L. Bender, .(1997) Plant growth promotion of wheat inoculated with Penicillium radicum sp. nov. Australian Journal of Soil Research, 35: 291-300.
52. Y.P. Chen, P.D. Rekha, A.B. Arun, F.T. Shen, W.-A. Lai, C.C. (2006). Young Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities *Applied Soil Ecology* 34:33–41.
53. Young, C.C, Chang, C.H., Chen, L.F., Chao, C.C. (1998), Characterization of the nitrogen fixing and ferric phosphate solubilizing bacteria isolated from Taiwan soil. J. *Chin. Agricult. Chem. Soc.* 36, 201–210 (in Chinese).