Research Article **ISSN 2250-0480** **Vol 2/Issue 3/Jul-Sept 2012**

International Journal of Life science & Pharma Research

MEDIA OPTIMIZATION FOR INORGANIC PHOSPHATE SOLUBILIZING BACTERIA ISOLATED FROM ANAND ARGICULTURE SOIL.

***AMIT SAGERVANSHI, ** PRIYANKA KUMARI, *ANJU NAGEE AND **ASHWANI KUMAR**

***Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences, ADIT Campus New V.V.Nagar, Anand, Gujarat.**
****Department of Botany, University of Rajasthan, Jaipur 302004, India**

ABSTRACT

Phosphorus (P) is one of the essential macronutrients for plant growth and reproduction. Plants acquire P from the soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca2+, Mg2+, Fe3+ and Al3+, depending on the particular properties of the soil and as a result, the phosphate is highly insoluble and unavailable to plants. Application of phosphate-solubilizing fungi (PSF) has been added as fertilizer to increase P uptake and plant growth. The improvement of soil fertility is one of the most common strategies to increase agricultural production. Maintaining high levels of available nitrogen (N) and phosphorus (P), the two most limiting nutrients in soil, remains big challenge. The present paper provides details about the different media used to get more phosphate solubilizing using isolated bacterial strains.

Key words: Phorphorus, biosolubilization, Orthophosphate. ATP.

INTRODUCTION

Phosphorous is second only to nitrogen as an essential macronutrient for plant growth and development (Scheffer*et al.,* 1998). Soil is rich in insoluble mineral and organic phosphates but deficient in available orthophosphate (Pi) (Dadarwal*et al.*, 1997). Phosphorus is a vital component of ATP, the "energy unit" of plants. ATP is formed during photosynthesis, has phosphorus in its structure. Soil amendment with phosphatic fertilizer, produced via chemical processing of rock phosphate ore, is therefore an absolute requirement in order to feed the world's population. For over one hundred years, workers have recognized the ability of soil microorganisms to solubilize Pi from insoluble (i.e. nutritionally unavailable) organic and mineral phosphates (*Whitelaw*, 2000). Wide ranges of microbial biosolubilization mechanisms exist, so that much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi. The genetic and biochemical mechanisms for this solubilization are as varied as the spectrum of P-containing soil compounds.

The limiting level of Pi in most soils provides the eco-physiological basis for positioning associations between plant roots and mineral phosphate solubilizing (MPS) and/or organic P solubilizing microorganisms. These associations are assumed to play an important role in phosphorus nutrition in many natural and agro-ecosystems. As a result, an enormous amount of research has been conducted involving isolation and characterization of MPS and organic P solubilizing microorganisms from a wide range of soils. In general, the goals

have been to understand P cycling and/or to develop P biofertilizers analogous to biological nitrogen fixation.

MATERIAL AND METHODS

COLLECTION OF SAMPLE

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

ISOLATION OF PSB

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 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.

OPTIMIZATION OF MEDIA AND GROWTH CONDITOIN FOR PHOSPHORUS SOLUBILIZATION

Phosphorus solubilizing ability of bacterial strains was tested in four different types of liquid media. Composition of different media is given in table 1. Rests of the experiment were performed in PVK media with 0.5% tricalcium phosphate. Flasks were inoculated with 8% (v/v) spore suspension and incubated on shaker at 28^{o}C for 6 days.

Table 1*: Composition of different media*

Media Component(g/l)	PVK	AYG	NBRIY	NBRIP
Glucose	10	20	10	10
$(NH_4)_2\ SO_4$	0.5	1.0	0.5	0.1
$MgSO_4.7H_2O$	0.1	0.5	0.1	0.25
Yeast Extract	0.5	0.2	_	_
KCl	0.2	_	0.2	0.2
NaCl	0.2	_	0.2	_
$FeCl_3$	_	Trace	_	_
$FeSO_4.7H_2O$	0.002	_	0.002	_
$MnSO_4.7H_2O$	0.002	Trace	0.002	_
$MgCl_2.6H_2O$	_	_	_	5.0
$Ca_3(PO_4)_5$	5	5	5	5.0
Ph	7.0	6.8	7.0	7.0

ESTIMATION OF PHOSPHORUS

Cultures were harvested after different growth periods in order to record the change in pH and concentration of P released in the medium. After centrifuging at 10,000 rpm for 15 min, the pH of the culture medium was measured with a pH meter equipped with a glass electrode. Dissolved phosphate concentration in the culture filtrate was determined by vanado-molybdate method as described in APHA (1995) it was expressed in terms of µg /ml phosphorus released in culture.

EFFECT OF VARIOUS PARAMETERS ON EFFICIENCY OF PHOSPHATE SOLUBILIZATION

Effect of various phosphorus source on efficiency of phosphate solubilization

Effect of various phosphorus sources like Alluminium phosphate, Zinc phosphate, Rock phosphate, Tricalcium phosphate were studied in PVK Broth (Nguye C et al 1992). The isolates were checked for solubilization activity in PVK broth amended with different phosphorus source. Inoculation was carried out by using pure colony of a bacterial. It was inoculated to PVK media and allowed to grow at 37°C, for 4 days respectively (Fasim et al., 2002). Turbidity in media was observed on second in bacterial culture. Efficiency of phosphate solubilization was calculated as mentioned above.

Effect of various Nitrogen sources on efficiency of phosphate solubilization

Effect of various Nitrogen sources like $(NH_4)_2SO_4$, Urea, Casein, and $NaNO_3$ were studied in PVK Broth. The isolates were checked for solubilization activity in PVK broth amended with different phosphorus source. Inoculation was carried out by using pure colony of a bacterial from LB Agar plate. Flask was incubated at 37°C for 4 days respectively (Fasim et al., 2002). Turbidity in media was observed on second in bacterial culture Efficiency of phosphate solubilization was calculated as mentioned above.

Effect of various Carbon sources on efficiency of phosphate solubilization

Effect of various carbon sources like glucose, fructose, sucrose, and lactose were studied in PVK Broth. The isolates were checked for solubilization activity in PVK broth amended with Tricalcium phosphate. Inoculation was carried out by using pure colony of a bacterial that had been grown, in LB Agar and PVK Agar at 37°C, 24 hrs and 28°C for 4 days respectively (Fasim et al., 2002). Efficiency of phosphate solubilization was calculated as mentioned above.

Effect of temperature on efficiency of phosphate solubilization

Media composition to which the bacteria responded best was used as substrate. Bacteria were inoculated and the culture was maintained at 30°C, 35°C, and 40°C and growth recorded as described above. Efficiency of phosphate solubilization was calculated.

Effect of pH on efficiency of phosphate solubilization

Optimal media and temperature was used , but the pH of the media was set at pH 5 , pH 6, pH 7 using NaOH or HCl and growth recorded as described above. Efficiency of phosphate solubilization was calculated as described above.

ORGANIC ACID PRODUCTION BY THE ISOLATES

For the organic acid determination by the isolates they were grown in PVK medium for 5 days at 28°C. On the sixth day the cultures were blended and were centrifuged at 5000 rpm for 10 min. Supernatant of each blended culture was filtered through 0.45 µm non sterile micro filter. 20 µl purified solution of each culture was injected in ion exchange column. The operating conditions consisted of 20µM NaH_2PO_4 the mobile phase at a constant flow rate of 1.0 ml/min and the column was operated at 30°C. The unknown organic acid in purified solution were determined by comparing the retention times and peak area of chromatograms with the standard of formic acid, oxalic acid, citric acid, acetic acid and succinic acid.

RESULTS & DISCUSSION

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**.

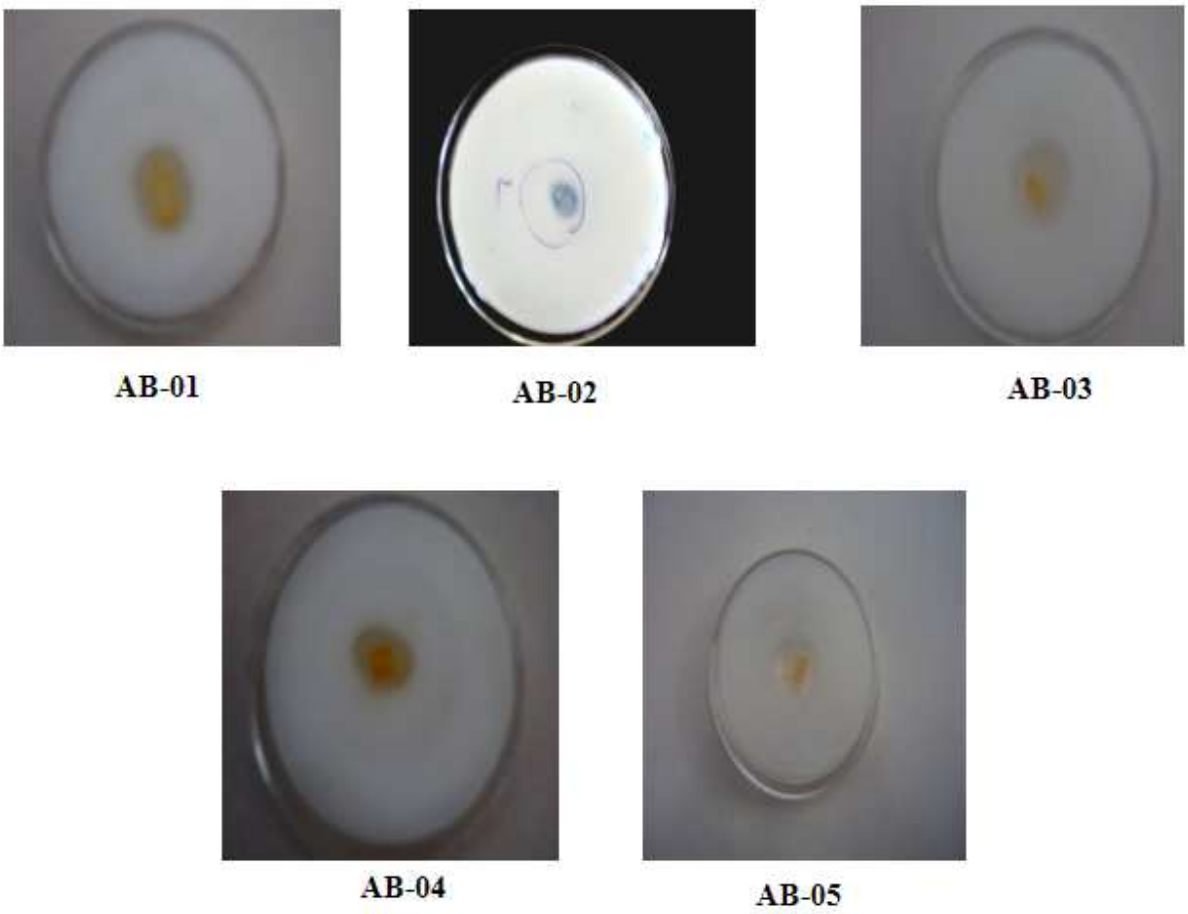


Figure 1: ***Halo Zone around the colony on PVK media confirm Phosphate solubilizing bacteria***

Optimization of different media for phosphorus solubilization:

After confirming the phosphorous solubilizing ability on solid medium the phosphorus solubilization in liquid medium was carried out. Different researchers have used different media for studding phosphorous solubilization in liquid medium. Aim was to find which media formulation is best for the isolates. The study was done using AB-01, AB-02,AB-03,AB-04 and AB-05. PVK (Pikovskaya, 1948) showed maximum P solubilization for all the three strain, maximum P solubilization was bone by AB-02, followed by AB-05 then AB-01S with resulting pH decrease, followed by NBRIY (Nautiyal, 1999) . AYG (Halder et al., 1991 and NBRIP (Nautiyal, 1999) showed low level of P solubilization. **Fig2A.**

pH is the vital factor in solubilization. Results show that with PVK there was fall in pH upto 4 and this was maximum among various

media used **Fig 2B**. In most of the cases P solubilization is the result of organic acid production although other mechanism many also be involved. Nahas 1996 showed that the solubilization of insoluble phosphate depends upon a multitude of factors including decrease in pH, microorganism and the insoluble phosphate used.

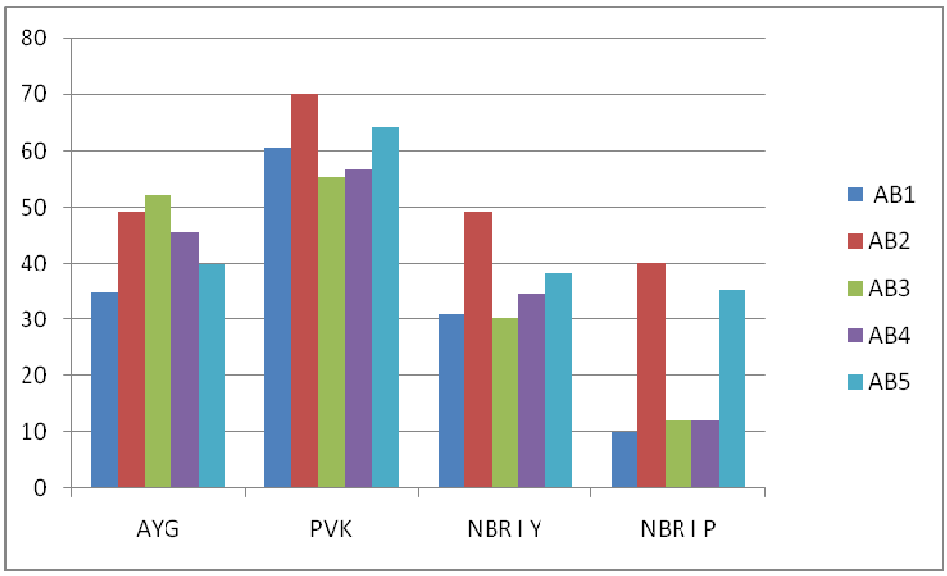


Figure 2A: ***Effect of various growth media on P Solubilization***

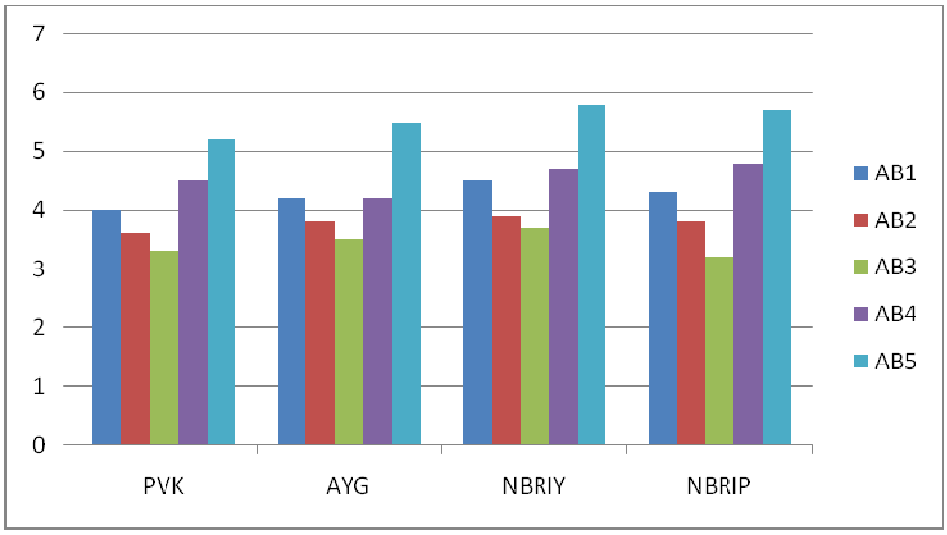


Figure 2B: ***Decrease in pH of various growth media.***

Effect of various phosphorus sources on efficiency of phosphate solubilization:

Effect of various phosphorus sources like Alluminium phosphate, Zinc phosphate, Rock phosphate, Tricalcium phosphate showed that tricalcium phosphate is the best phosphate source and that maximum fall was seen i.e pH 3.8 in case of AB-02 isolate. Next was alluminium phosphate which showed medium P solubilization whereas Zink and rock phosphate showed very less P solubilization. With rock phosphate maximum pH fall was seen up to pH 2.9 in case of AB-04. Metals

ion is made available to soil after the metal ion bound phosphate. Metal ion are consider to be macro element needed for plant growth (Agnihorti, 1970).

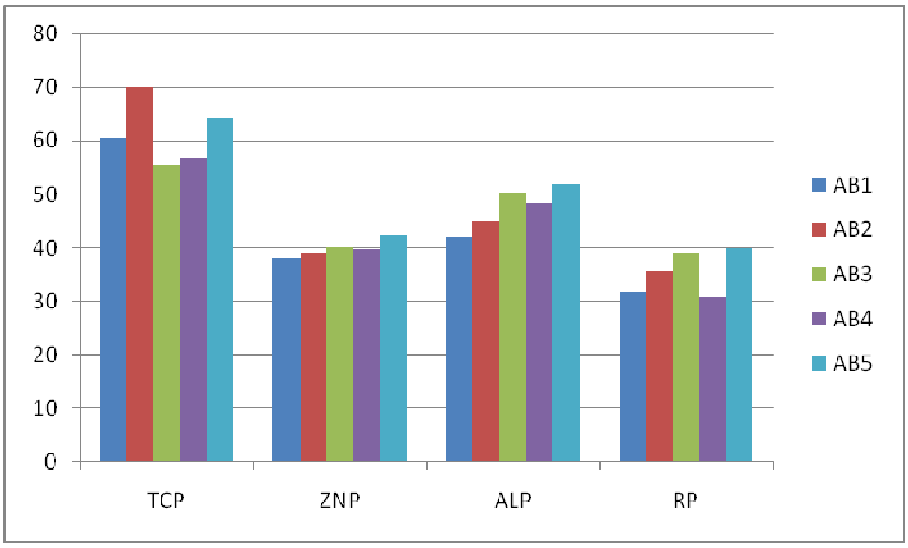


Fig 3: ***Effect of various phosphorus sources on P solubilization***

Effect of various Nitrogen sources on phosphate solubilization:

While studying the effect of various nitrogen sourced on the phosphate solubilization it was found that $(NH_4)_2SO_4$ showed maximum P solubilization followed by Casein. Urea and $NaNo_3$ gave very less P solubilization. In control with no nitrogen source substantial growth was there and drop of pH was there and little solubilization of P which may be due to yeast extract and glucose in medium which was utilized by bacteria as nitrogen source. A number of fungi and bacteria have been reported of being able to solubilized phosphate only in the presence of ammonium as the nitrogen sources (Illmer and schinner, 1992; Lapeyrie, 1991). Nitrogen source in form salt seems to be important as it increase phosphorous solubilization of rock phosphate (Asea, 1988).

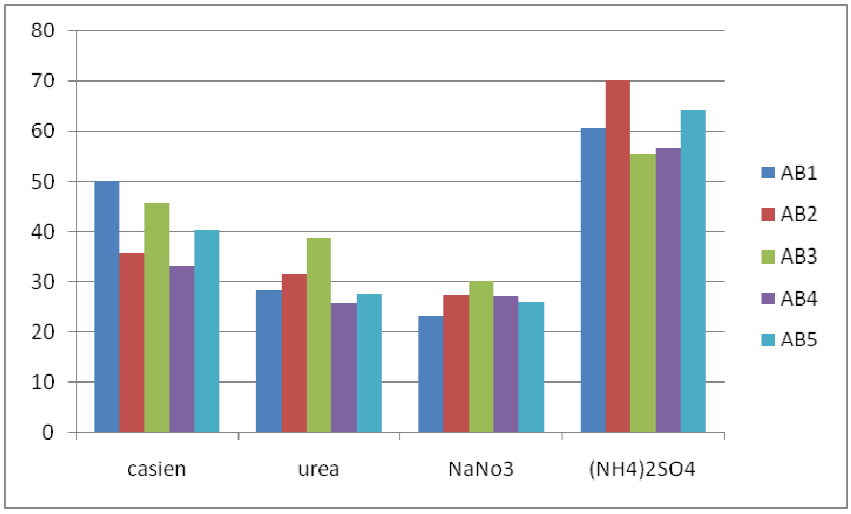


Fig4: ***Effect of various Nitrogen sources on P solubilization***

Effect of various Carbon sources on phosphate solubilization

When various carbon sources were used to study the P solubilization it was found that PVK with glucose gave maximum P solubilization with a fall in pH to 4.2 followed by galactose. In control without glucose some growth was seen due to presence of yeast extract in the medium, but drop in pH and P solubilization was quite low. The solubilization ability of microorganism is related to its organic acid production; however nature of acid produced is also important (Vassileva, 1998). Fasim et al. 2002 have reported bacterial isolates which solubilize P only in presence of glucose and while co solubilization in presence of gluconate, galactose and fructose.

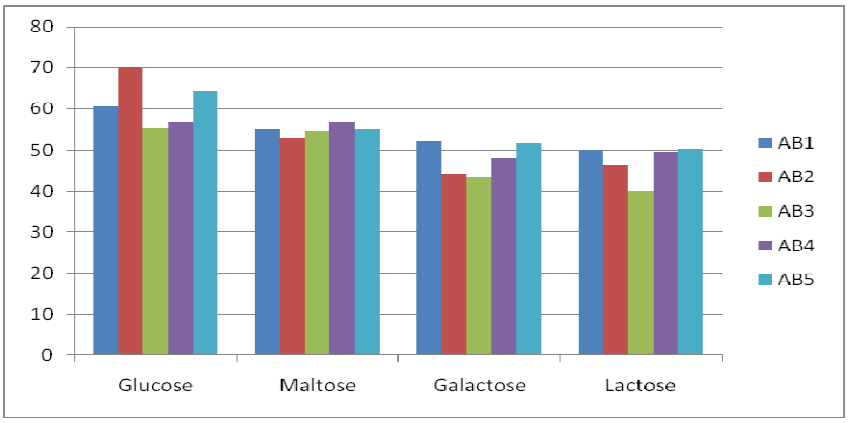


Figure 5: ***Effect of various carbon sources on P solubilization.***

Effect of temperature on efficiency of phosphate solubilization:

For all the isolates 35°C temperature is the optimum temperature for the growth and phosphate solubilization. P solublization was also seen at 30°C and it was found that after 35°C there was growth retardation and fall in P solublization. Different temperature have been reported by earlier workers for solubilization , most of them have found 25°C to 28°C to be optimum temperature (Sayer and Gadd, 1998). There are reports that have shown P solublization at 45°C and some at 10°C. This shows that bacteria adapt to their indigenous environment so their metabolic activites are linked to the temperature of the environment. ([SadafShahab, 2008,Varsha NHH (2002)]

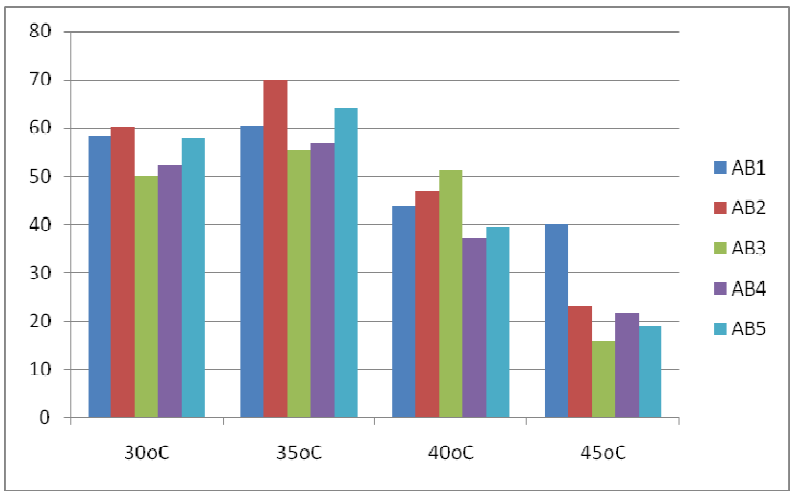


Figure 6: ***Effect of Temperature on P solubilization.***

Effect of pH on efficiency of phosphate solubilization:

Results show that all the isolates were able to P solubilize in the pH range of 5 to 7. Maximum P solublization and growth was monitored at pH 7. At the same time retardation in growth and P solublization was observed at pH 8. Acid predation has been reported to be a major mechanism involved in solubilization. pH is the vital factor in solubilization , in most of cases P solublization is the result of organic acid production. There are also other mechanisms such as production of bacterial metabolites and siderophores have also attributes to solubilization(Sadaf Shahab, 2008)

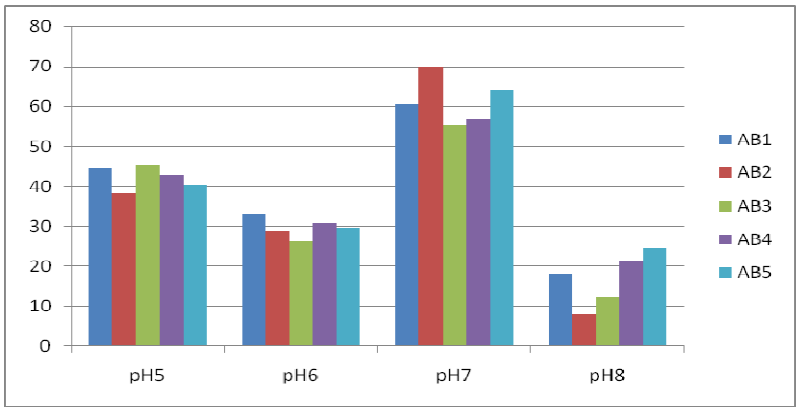


Figure 7: ***Effect of pH on P solubilization***

Analysis of organic acid produced by HPLC

HPLC analysis showed that formic acid as main acid produced by AB-01, AB- 02,AB-04,and AB-05 where as AB-03 showed succinic acid and citric acid. It is been reported that solublization of phosphate is mediate by organic acid produced by PSM (Cuningham J E 1992)

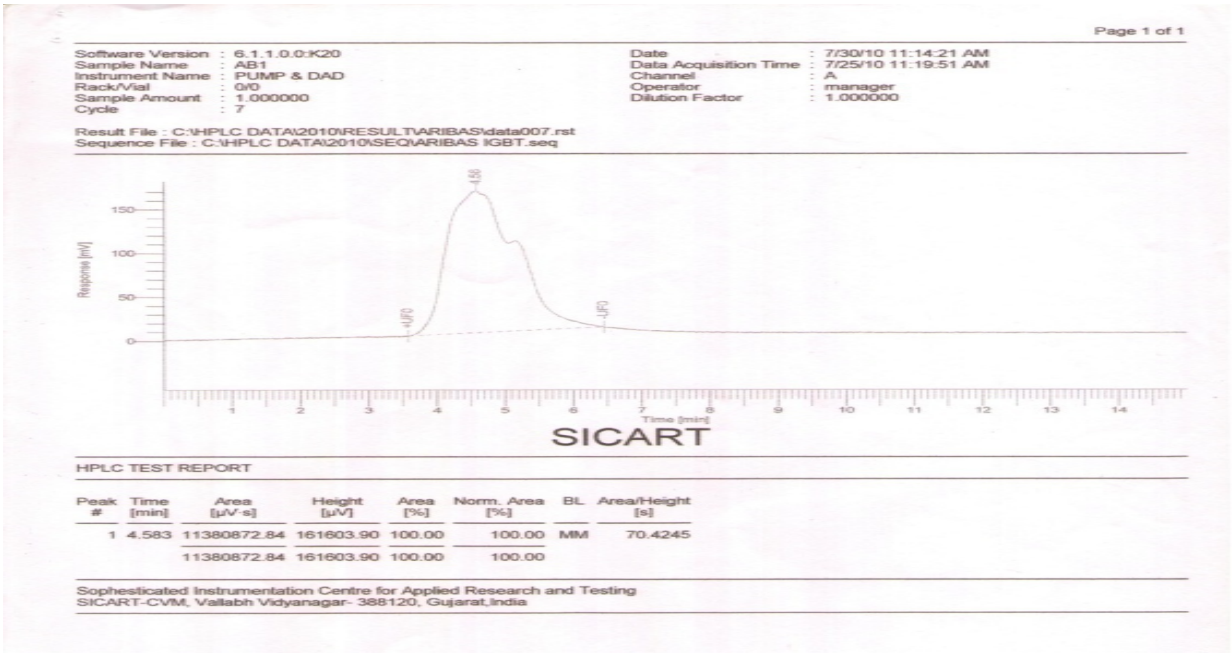
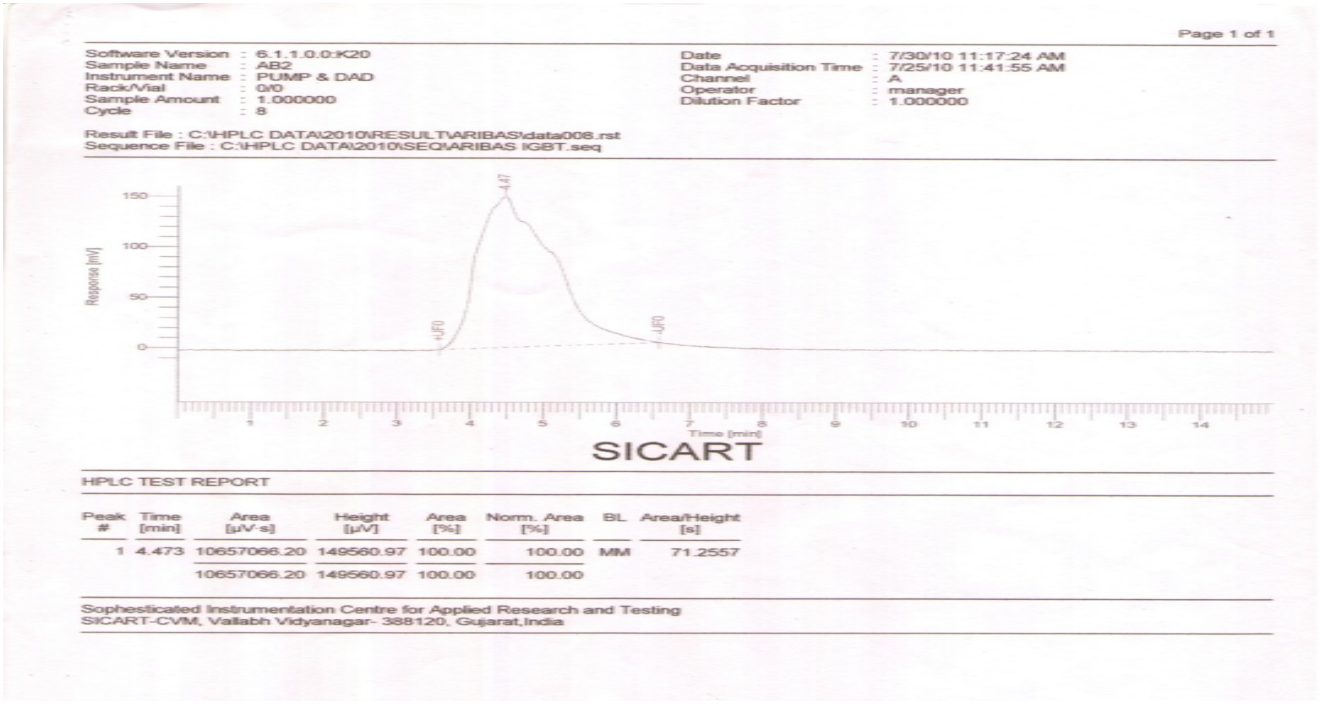


FIGURE-*AB-01*



Page 1 of 1

Software Version : 6.1.1.0.0:K20
Sample Name : AB2
Instrument Name : PUMP & DAD
Rack/Vial : 0/0
Sample Amount : 1.000000
Cycle : 8

Date : 7/30/10 11:17:24 AM
Data Acquisition Time : 7/25/10 11:41:55 AM
Channel : A
Operator : manager
Dilution Factor : 1.000000

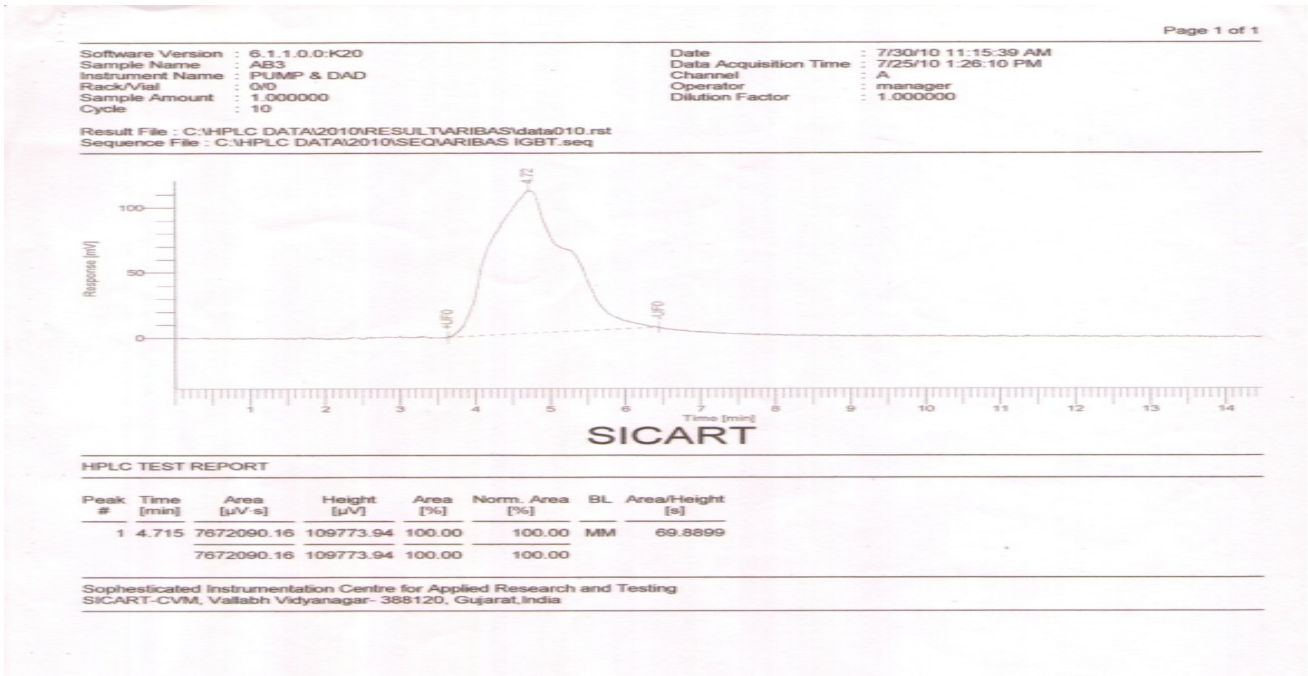
Result File : C:\HPLC DATA\2010\RESULT\ARIBAS\data008.rst
Sequence File : C:\HPLC DATA\2010\SEQ\ARIBAS IGBT.seq

HPLC TEST REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	4.473	10657066.20	149560.97	100.00	100.00	MM	71.2557
		10657066.20	149560.97	100.00	100.00		

Sophesticated Instrumentation Centre for Applied Research and Testing
SICART-CVM, Vallabh Vidyanagar- 388120, Gujarat,India

Figure-*AB-02*



Page 1 of 1

Software Version : 6.1.1.0.0:K20
Sample Name : AB3
Instrument Name : PUMP & DAD
Rack/Vial : 0/0
Sample Amount : 1.000000
Cycle : 10

Date : 7/30/10 11:15:39 AM
Data Acquisition Time : 7/25/10 1:26:10 PM
Channel : A
Operator : manager
Dilution Factor : 1.000000

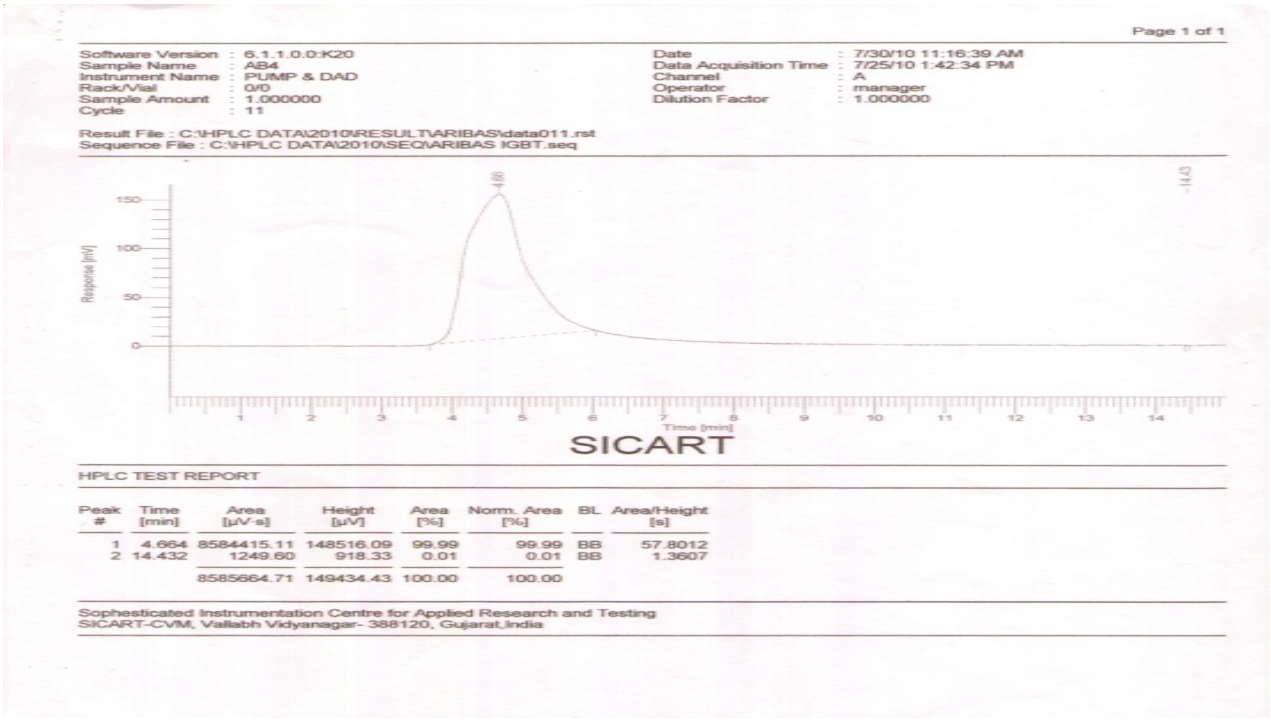
Result File : C:\HPLC DATA\2010\RESULT\ARIBAS\data010.rst
Sequence File : C:\HPLC DATA\2010\SEQ\ARIBAS IGBT.seq

HPLC TEST REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	4.715	7672090.16	109773.94	100.00	100.00	MM	69.8899
		7672090.16	109773.94	100.00	100.00		

Sophesticated Instrumentation Centre for Applied Research and Testing
SICART-CVM, Vallabh Vidyanagar- 388120, Gujarat,India

Figure-AB-03



Page 1 of 1

Software Version : 6.1.1.0.0:K20
Sample Name : AB4
Instrument Name : PUMP & DAD
Rack/Vial : 0/0
Sample Amount : 1.000000
Cycle : 11

Date : 7/30/10 11:16:39 AM
Data Acquisition Time : 7/25/10 1:42:34 PM
Channel : A
Operator : manager
Dilution Factor : 1.000000

Result File : C:\HPLC DATA\2010\RESULT\ARIBAS\data011.rst
Sequence File : C:\HPLC DATA\2010\SEQ\ARIBAS IGBT.seq

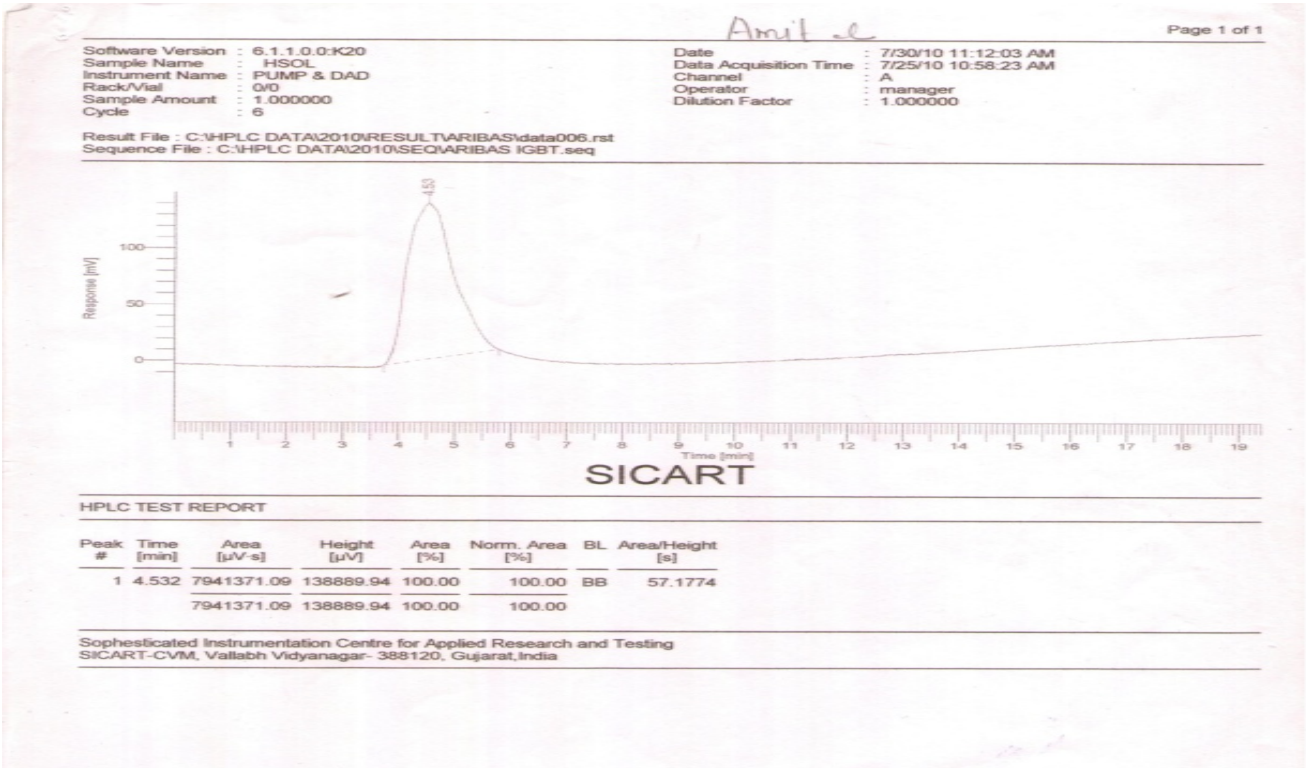
SICART

HPLC TEST REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	4.664	8584415.11	148516.09	99.99	99.99	BB	57.8012
2	14.432	1249.60	918.33	0.01	0.01	BB	1.3607
		8585664.71	149434.43	100.00	100.00		

Sophesticated Instrumentation Centre for Applied Research and Testing
SICART-CVM, Vallabh Vidyanagar- 388120, Gujarat,India

Figure-*AB-04*



Amit .c

Page 1 of 1

Software Version : 6.1.1.0.0:K20
Sample Name : HSOIL
Instrument Name : PUMP & DAD
Rack/Vial : 0/0
Sample Amount : 1.000000
Cycle : 6

Date : 7/30/10 11:12:03 AM
Data Acquisition Time : 7/25/10 10:58:23 AM
Channel : A
Operator : manager
Dilution Factor : 1.000000

Result File : C:\HPLC DATA\2010\RESULT\ARIBAS\data006.rst
Sequence File : C:\HPLC DATA\2010\SEQ\ARIBAS IGBT.seq

SICART

HPLC TEST REPORT

Peak #	Time [min]	Area [µV·s]	Height [µV]	Area [%]	Norm. Area [%]	BL	Area/Height [s]
1	4.532	7941371.09	138889.94	100.00	100.00	BB	57.1774
		7941371.09	138889.94	100.00	100.00		

Sophesticated Instrumentation Centre for Applied Research and Testing
SICART-CVM, Vallabh Vidyanagar- 388120, Gujarat,India

Figure-*AB-05*

REFERENCES

1. Scheffer F, Schachtschabel P. Lehrbuch der Bodenkunde. Stuttgart: Enke, (1998).The role of rock *phosphate solubilizing* fungi and vesicular arbuscularmycorrhiza (VAM)
2. 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.
3. 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.
4. 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.
5. 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.
6. Illmer P, Schinner F. (1992). Solubilization of hardly-soluble AlPO4 with P-solubilizing microorganisms.*Soil Bio and Biochem*. 24: 389-395.
7. 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.
8. 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
9. 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.
10. 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.
11. 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.
12. *Agnihotri* V P, (1970) Solubilization of insoluble phosphate by some soil fungi isolated from nursery seedbeds. Can. J. Micro.16,877-880.
13. 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.
14. 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.