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Solubilisation of inorganic phosphates by fungal and actinomycete isolates from soil of Bihar

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Abstract: Phosphate availability in soil is limiting factor for the growth of plants. Phosphate solubilising microbes can be implicated as biofertilizers. The phosphate solubilizing microorganism was initially isolated on basic growth medium for fungi and actinomycetes. The screening for phosphate solubilisation of isolates was carried on Pikovskays (PVK) and National Botanical Research Institute phosphate (NBRIP) medium supplemented with 0.5% (W/W) calcium phosphate as the substrate. The effect of different phosphate sources [$Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and rock phosphate as control] and their concentration, incubation time, temperature and changes in pH of media after the solubilisation of phosphate was analyzed. The screening for phosphate solubilisation potential was based on holo-zone formation on solid medium, while soluble phosphate in the culture filtrate was determined by vandomolybdate method. The isolates *Aspergillus niger* US11 and *Aspergillus terreus* US3 could solubilize maximum TCP at a temperature of 30ºC while *Penicillium* sp. US10 solubilize maximum TCP at 25ºC. On the other hand the both the potential actinomycetes isolates solubilise TCP at a higher temperature of 35ºC. All the fungal isolates showed maximum solubilization of TCP at pH 7 whereas in case of actinomycetes pH 8 was optimum for solubilization. After 144 hrs of growth, maximum solubilisation of TCP equivalent to 1353 mg/ L was demonstrated by the isolate *Aspergillus niger* US11 when the initial TCP concentration of 7500 mg/L was used in the medium. *Aspergillus terreus* also showed maximum solubility at the initial concentration of 7500 mg/L, however the growth time was only 96 hrs. The other three strains demonstrated maximum solubilisation of TCP when the initial concentration of TCP was kept at 5000mg/L in the medium. However Actinomycetes sp. reached maximum solubility of phosphate at relatively less time of only 72 hrs. Our finding suggested that all selected isolates can be used for the preparation a biofertilizer for the different crops.

Index Terms **– Phosphate solubilizing microbes, biofertilizer, temperature.**

I. INTRODUCTION

Phosphorus is essential element that is needed for plant development and growth; about 0.2 % of plant dry weight is made up of Phosphorous. It is second most important mineral nutrients most commonly limiting the growth of crops (Azziz et al., 2012; Tak et al., 2012). Although in soil the phosphorus content is about 0.05% (w/w); only 0.1% of this phosphorus is available for plant use (Zhu et al., 2011). For the deficiency of phosphorous chemical fertilizers are utilized, however, the majority of the phosphorus in fertilizer is not available to plants and the addition of inorganic fertilizers in excess can lead to environmental problems including groundwater contamination and eutrophication of waterway (Kang et al., 2011). Soil microorganisms enhance plant nutrient acquisition and some are capable of solubilizing and mineralizing insoluble soil phosphorus for the growth of plants. In soil and rhizosphere numerous microorganisms are effective in releasing phosphorus from total soil phosphorus through solubilization and mineralization processes (Bhattacharyya and Jha, 2012). These microorganisms are called Phosphorus Solubilizing Microorganisms (PSM). Many species of soil fungi and bacteria are able to solubilize phosphorus *in vitro* and some of them can mobilize phosphorus in plants (Zhu et al., 2011). PSM increases the bioavailability of soil insoluble phosphorus for plant use (Zhu et al., 2011). The inoculation of soil or crop with phosphate solubilizing/mineralizing microorganisms is a promising approach for the increase of plant absorption of phosphorus and which reduce the use of chemical fertilizers (Alori et al., 2012).

Phosphorus Solubilizing Microorganisms (PSM)

A large number of microbial organisms including bacteria, fungi, actinomycetes, and algae exhibit P solubilization and mineralization ability. In addition to numerous soil bacteria, fungi including strains of *Achrothcium, Alternaria, Arthrobotrys, Aspergillus, Penicillium, Pythium, Rhizoctonia, Rhizopus, Saccharomyces, Schizosaccharomyces, Schwanniomyces, Sclerotium, Torula, Trichoderma*, and *Yarrowia* have the ability to soulbilize and mineralize phosphates (Srinivasan et al., 2012; Sharma et al., 2013). Soil fungi have are able to cross long distances more easily than bacteria and are considered as more important in the solubilization of inorganic phosphate in soils as they have the ability to produce and secrete more acids, like gluconic, citric, lactic, 2-ketogluconic, oxalic, tartaric and acetic acid, than bacteria (Sharma et al., 2013). Further, approximately 20% of actinomycetes have been believed to solubilize phosphorous, including those in the genera *Actinomyces, Micromonospora*, and *Streptomyces* (Sharma et al., 2013). Phosphorus is treated by PSM in three different ways namely, solubilization, mineralization and immobilization. Insoluble phosphates are dissolved by acidification, chelating or proton extrusion mechanisms. Acidic solubilization mainly concerned with the acidification of growth medium by the production of organic and inorganic acids. Acid production in laboratory cultures is indicated by decrease in pH of growth medium. Chelation process is also one of the important phosphate solubilization processes. Organic form of phosphatic compounds is transformed into utilizable form by PSM via process of mineralization. *Bacillus* spp. and *Streptomyces* spp. are able to mineralize very complex organic phosphate by production of extracellular enzymes like phosphoesterase, phosphodiesterases, phytase and phospholipases (Molla *et al*, 1984).The aim of the present study was to screen the efficient phosphate solubilizers and to optimize the conditions such as substrate concentration, temperature, pH and other parameters for maximum solubilization.

2. Materials & Methods

2.1 Isolation, Preliminary Screening and identification: Isolation of soil fungi and actinomycetes was done by erial-dilution-spread plate technique using solidified potato dextrose agar (PDA) and starch casein agar (SCA) plates. PDA media was supplemented with antibiotic streptomycin at a concentration of 250µg/ml to inhibit the bacterial growth. SCA plates were incubated at 35±1ºC whereas PDA plates were incubated at 28±1ºC. After 3–4 days, plates were observed for the growth of microbes and pure cultures were obtained. Preliminary screening for phosphate solubilising ability of isolates was carried out by examining the "halos"formed on solid agar plates and broth containing Pikovskaya (pvk) (Pikovskaya,1998)and national botanical research institute phosphate (NBRIP) medium (Nautiyal, 1999) supplemented with 0.5% (w/w) calcium phosphate as the substrate. Identification of fungi and actinomycetes was based upon their colony morphology, spore characteristics and microscopic studies.

2.2 Preparation of inoculum and *Estimation of phosphate solubilization*:

50 ml sterile pvk broth in 250ml Erlenmeyer flasks containing tri-calcium phosphate (5.0gm/L) as P-source at pH 6.8±2 was prepared. The broth was inoculated with a 4-days old culture spore density of 10^6 spores/ ml incubated for 196 hours at 30 ± 0.2^0c and shaken at 120 rpm. For the effect of different phosphate source on the solubilisation Ca3(PO4)2, AlPO4, FePO4 and rock phosphate as control was used. P solublizing capacity of the isolates was determined after different time periods. Culture medium was centrifuged at 8000 RPM for 15 min and the supernatant was assayed for solubilized phosphorous in the medium by vanado-molybate method as described in APHA (1995). It was expressed released in the term of mg/L phosphorus solubilized in the culture medium.

3. RESULTS

3.1 Effect of different tricalcium phosphate concentration on solubilization during broth culture:

The *A. Niger* US11 produced maximum soluble phosphate (1353±21 mg/L) after 144 hours of incubation when concentration of tri-calcium phosphate (TCP) was 7500mg/L. The isolate *A. terreus*US3 also produced maximum soluble phosphate (1185±71mg/L) in the same concentration of TCP but within 96 hours of incubation (Table-1). In case of *Penicillium* sp.US10 5000mg/L of TCP was optimum for soluble phosphate production within 96 hours of incubation. With regard to actinomycetes strains (*Streptomyces* sp.US16 and *Streptomyces* sp. US5), maximum soluble phosphate produced (1192±14mg/L and 1122±14mg/L) within 72 hours of incubation when TCP concentration was 5000 mg/L in the medium.Therefore, among the fungal strains, *A. niger* US11 was found to be most efficient phosphate solubilizer, while in case of actinomycetes, the strain *Streptomyces* sp.US5 was found to be very efficient because it carried out the solubilization of TCP within 72 hours (Table-1)

3.2 Effect of pH on solubilization of tri-calcium phosphate [Ca_3 (PO_4)$_2$] by selected strains: All selected strains were inoculated separately in PVK media (fungi) and modified SCA media (for actinomycetes) containing tri-calcium phosphate (TCP) having different pH (pH 5 to pH 9) and incubated at suitable temperatures in shake culture condition (Table-2). All fungal strains showed growth and maximum TCP solubilization at pH 7. At low and high pH, growth of organisms and solubilization of tricalcium phosphate showed a decreasing trend. In case of

actinomycetes, both selected strains showed good growth and phosphate solubilization at slightly alkaline pH. During present investigation pH 8 was found to be optimum for growth and solubilization of TCP (Table-2).

3.3 Solubilization of different phosphate sources by selected strains:

All selected strains were inoculated separately in PVK media (for fungi) and modified SCA media (for actinomycetes) with 5gm/L of tricalcium phosphate [$Ca_3(PO_4)_2$], aluminium phosphate ($AlPO_4$), iron phosphate ($FePO_4$) and rock phosphate in separate flask and incubated at suitable temperature on shaking condition (120rpm) in dark. Uninoculated flasks for each source were kept as control under same condition. After 72 hours, soluble phosphorus in the culture filtrate was determined by vandomolybate method. All tested strains, except *Penicillium* sp. US10 converted insoluble ferric phosphate ($FePO_4$) into soluble forms very efficiently .Among different strains, *A. niger* US11 was found to be the most efficient strain for solubilization of majority of phosphate substrates and maximal solubilization occurred in case of $FePO_4$ substrate (1598 mg/L). *A. terreus*US3 also solubilized $FePO_4$ very efficiently. Actinomycetes strains also able to solubilized $FePO_4$ efficiently. *Penicillium*sp.US10 did not solubilize $FePO_4$ efficiently. But this strain solubilized aluminium phosphate more efficiently (1028mg/L) within 72 hours of incubation (Fig-1). On the basis of screening with four different substrates, *A. niger*US11 was able to solubilize all substrates significantly except rock phosphate. With regard to rock phosphate and calcium phosphate $Ca_3(PO_4)_2$, *Streptomyces* sp.US16 was found to be a very efficient solubilizer (Fig.1)

3.4 Effect of temperature on solubilization of tricalcium phosphate:

All selected strains were inoculated separately in PVK media (for fungi) and SCA media (for actinomycetes) containing tricalcium phosphate (TCP) and incubated at different temperatures (20-40ºC) in shake culture condition. For fungal strains *A. Niger* US11 and *A. terreus*US3, a temperature of 30ºC was found to be optimum for the growth and solubilization of TCP and 25ºC was most favourable for *Penicillium*sp.US10 (Fig.2). At higher temperatures (35ºC to 40ºC), the growth and solubilization drastically reduced. In case of actinomycetes, both strains showed good growth and solubilization of TCP at higher temperatures and maximum solubilization achieved at 35ºC (Fig-2).

3.5 Effect of pH on the solubilisation of tri-calcium phosphate:

The maximum TCP solubilization occurred at pH range 6.0-7.0 in all three fungal strains. In actinomycetes strains, optimum solubilization was achieved at pH range 7.0-8.0 (Fig.3).

DISCUSSION:

In the present study fungal and actinomycetes were isolated from soil with the aim to find the phosphate solubilizing microbes. From the isolates, altogether three fungal and two actinomycetes showed potential phosphate solubilizing activity. A large number of fungi and actinomycetes exhibiting P solubilization and mineralization abilities have been isolated from soil (Srinivasan et al., 2012; Sharma et al., 2013). Soil fungi are able to cross long distances more easily than bacteria and are considered as more important in the solubilization of inorganic phosphate in soils as they have the ability to produce and secrete more acids, like gluconic, citric, lactic, 2-ketogluconic, oxalic, tartaric and acetic acid, than bacteria (Sharma et al., 2013). Further, approximately 20% of actinomycetes have been believed to solubilize phosphorous, including those in the genera *Actinomyces*, *Micromonospora*, and *Streptomyces* (Sharma et al., 2013).

In present study the soil fungi and actinomycetes were able to solubilise phosphate. A number of theories explain the mechanism of inorganic phosphate solubilization. As observed in many experiments, the principal mechanism is the production of mineral dissolving compounds such as organic acids, siderophores, protons, hydroxyl ions and CO2 (Cunningham and Kuiack, 1992; Rodríguez and Fraga, 1999; Sharma et al., 2013). The insoluble forms of P are solubilized by P-solubilizing microorganisms either by (i) lowering the pH or (ii) by enhancing chelation of the cations bound to P (Omar, 1998; Whitelaw, 2000; Maliha *et al*, 2004). In case of fungal strains, solubilization of tricalcium phosphate continued with the decrease in pH of the medium and after the peak of the solubilization, the pH of the medium slightly increased. The lowering in pH of the medium suggested the release of organic acids by the fungal strains (Whitelaw, 2000; Maliha *et al*, 2004). The synthesis and discharge of organics acids by the fungal strains acidify the surrounding environment and ultimately lead to the release of P ions from the mineral by H^+ substitution for Ca^{2+} (Goldstein, 1994). After the maximum solubilization of phosphate, the pH slightly increased. It might be due to consumption of acids by the microorganisms for their own growth (Whitelaw *et al*, 1999). Vassilev *et al*, (1995) also observed a decrease in acid concentration during solubilization of rock phosphate by *A. niger*. It was probably due to citric acid utilization by fungus under conditions of nutrient depletion. Similar finding was also observed in *Penicillium radicum* by Whitelaw *et al*, (1999).

In case of actinomycetes strains, solubilization of tricalcium phosphate carried out with the increase in pH of the medium with maximum value of pH 8.5. In these cases, solubilization of P occurred without acid production. It might be due to the release of proton accompanying respiration or ammonium assimilation (Kucey, 1983; Dighton

and Boddy, 1989) and by chelation of the cation bound to P (Whitelaw, 2000). Similar observation has also been recorded in case of fast growing *Azospirillium brasilense* (Rodiquez *et al*, 2004) and in case of fungus *Ustilago* sp. (Paul and Clark, 1996).

To know the impact of concentration of TCP on solubilization, suitable amount of TCP (to obtain a concentration range of 2500 mg/L to 10,000 mg/L) was added in PVK (for fungi) and SCA (for actinomycetes) media. Media were inoculated with suitable strains and incubated at appropriate temperature. The result of present investigation suggested the decrease in phosphate solubilization at higher TCP concentration. In case of *A. niger* US11 and *A. terreus* US3 the optimum solubilization of TCP occurred at 7500mg/L concentration. Whereas in *Penicillium* sp.US10 and both actinomycetes strains (US5 and US16), the optimum solubilization occurred at 5000 mg/L concentration. Similarly, Gothwal *et al*, (2006) also observed the optimum phosphate solubilization by four rhizospheric isolates (strain T-1, TS-11, T-3 and TS-18) within the range of 5000 mg/L to 10000 mg/L. Chai *et al*, (2011) also observed the decrease of phosphate solubilization efficiency above 10,000mg/L TCP concentration by *Penicillium* sp.

Our results showed that the optimum temperatures for P solubilization by *A. niger* US11 and *A. terreus* US3 were 30ºC whereas maximum P solubilization by *Penicillium* sp. US10 was carried out at 25ºC.Optimization of temperature is one of the important parameters for solubilization of tricalcium phosphate and it has been suggested that 35ºC for optimum solubilization of TCP in case of *Aspergillus awamori* (Jain *et al*, 2011) whereas Pandey *et al*, (2008) reported a temperature ranges from 21 to 28ºC for optimum solubilization for *Penicillium* sp. In case of actinomycetes the optimal temperature for solubilization of TCP by both strains of actinomycetes was 35°C. Variation among the temperature optima of different isolates might be due to their morphological, physiological and metabolic differences.

Besides temperature, pH of the media also influenced the solubilization of TCP. During present investigation, maximum TCP solubilization occurred at pH range 6.0-7.0 in all three fungal strains. In actinomycetes strains, optimum solubilization was achieved at pH range 7.0-8.0. Similar findings were observed by Wani *et al*, (1979) in case of *Aspergillus awamori*, *Penicillium digitatum*, *Pseudomonas striata* and *Bacillus polymyxa*. Jain *et al* (2011) observed optimum solubilization of TCP at pH 8.0 in case of *A. awamari* S19. Scervino *et al*, (2011) observed that the solubilization efficiency was lowest at high pH in case of *Penicillium purpurogenum*.

To know the impact of solubilization efficiency of different P substrates, the solubilization of different forms of phosphates such as calcium (Ca), aluminium (Al), ferric (Fe) and rock phosphate was also carried out. Generally Al and Fe phosphates have been less solubilized than calcium phosphate, and solubility of aluminium phosphate has been greater than that of iron phosphate (Gadagi and Tangmin, 2002). Our investigation showed that the *A. niger* US11 and *A. terreus* US3 were very good solubilizers of $FePO_4$. *Penicillium* sp. US10 efficiently solubilized $AlPO_4$. Both *Streptomyces* spp. were capable to solubilize different phosphate sources in following order: *Streptomyces* sp. US16- Ca-P > Rock-P> Fe-P > Al-P and *Streptomyces* sp. US5- Fe-P > Ca-P ≈ Al-P > Rock P. All three fungal strains showed less solubility of rock phosphate. Most studied on microbial solubilization of phosphates are confined to solubilization of tricalcium phosphate or hydroxyapatite, which are solubilized more easily than rock phosphate (Gaur, 1990). Gaur and Gaind (1983) also investigated significant solubilization of ferric and aluminium phosphates in liquid medium by *Aspergillus awamori*. The *Pseudomonas striata* able to solubilized tricalcium, aluminium and iron phosphates. Our results also showed that all selected strains are capable to solubilize tricalcium, iron and aluminium phosphates very effectively.

On the basis of overall findings of the present investigation, all selected strains showed good aptitude to solubilize both insoluble organic and inorganic phosphates. Based on the phosphate solubilising activity of the strains it is proposed that they may be developed as biofertilizers for the staple crops.

Figures and Tables

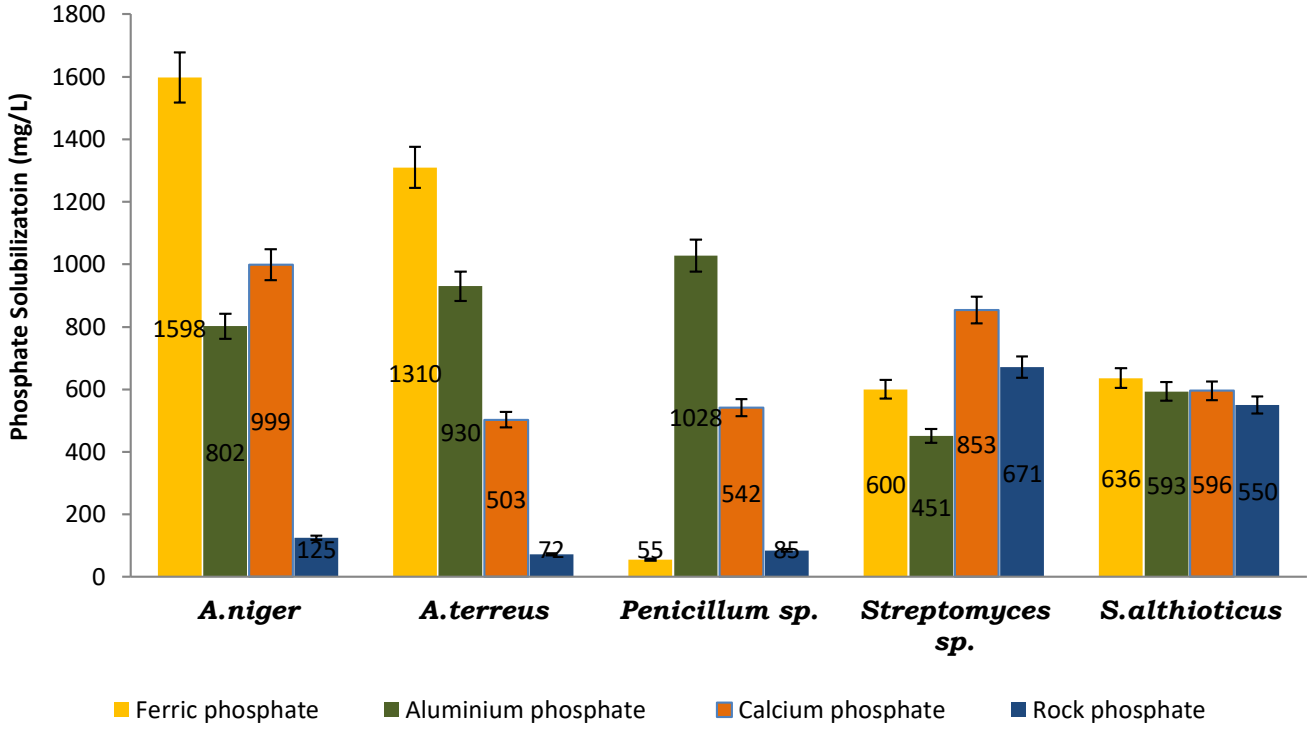

Fig.1. Solubilization of different phosphate sources by selected strains *A. niger* US11, *A.terreus* US3, *Penicillium* sp.US10, *Streptomyces* sp.US16, *S. althioticus* US5.

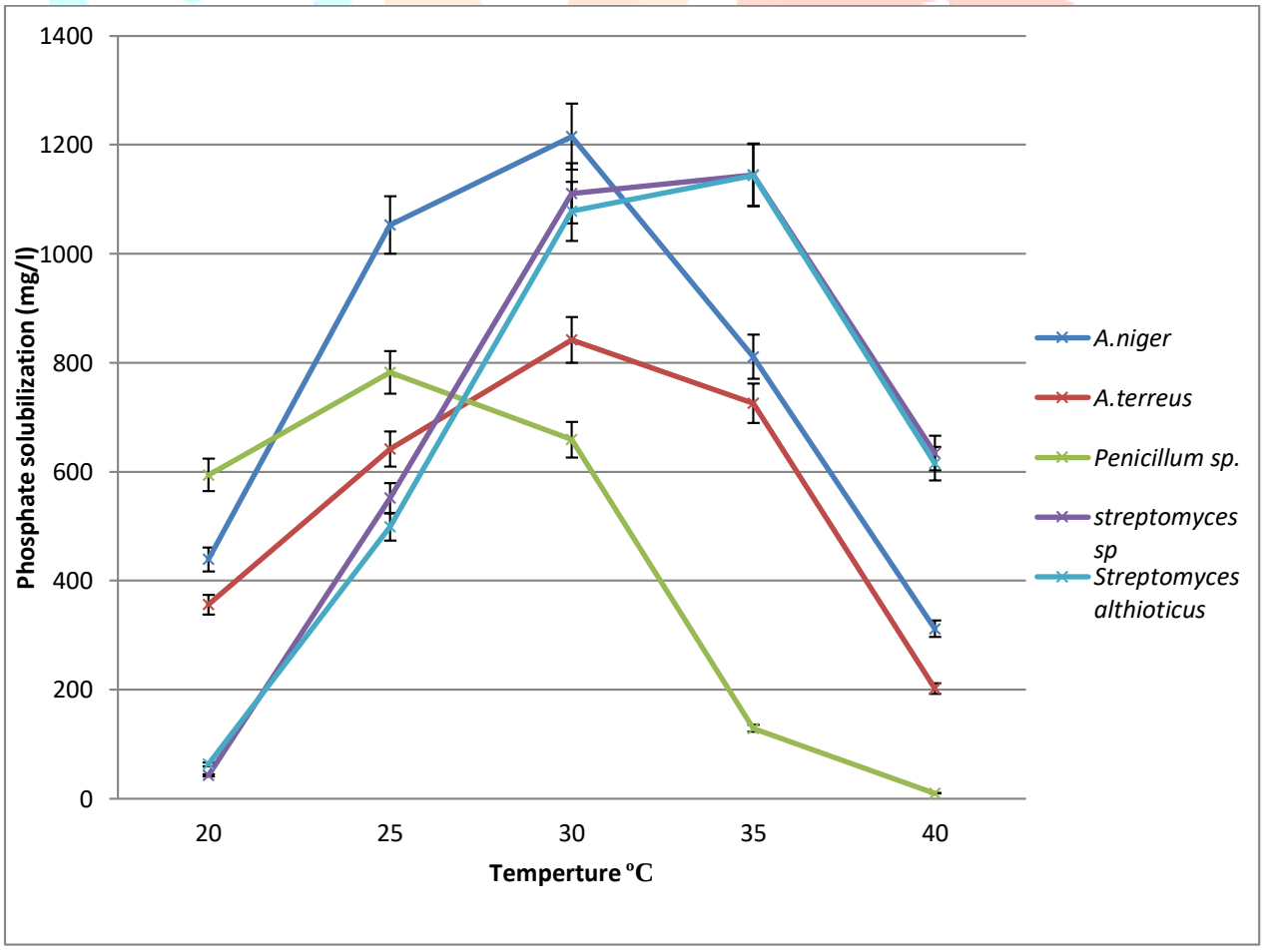

Fig.2. Effect of temperature on solubilization of $Ca_3 (Po_4)_2$ by selected Strains *A.niger* US11, *A. terreus* US3, *Penicillium* sp.US10, *Streptomyces* sp.US16, *S.althioticus* US5.

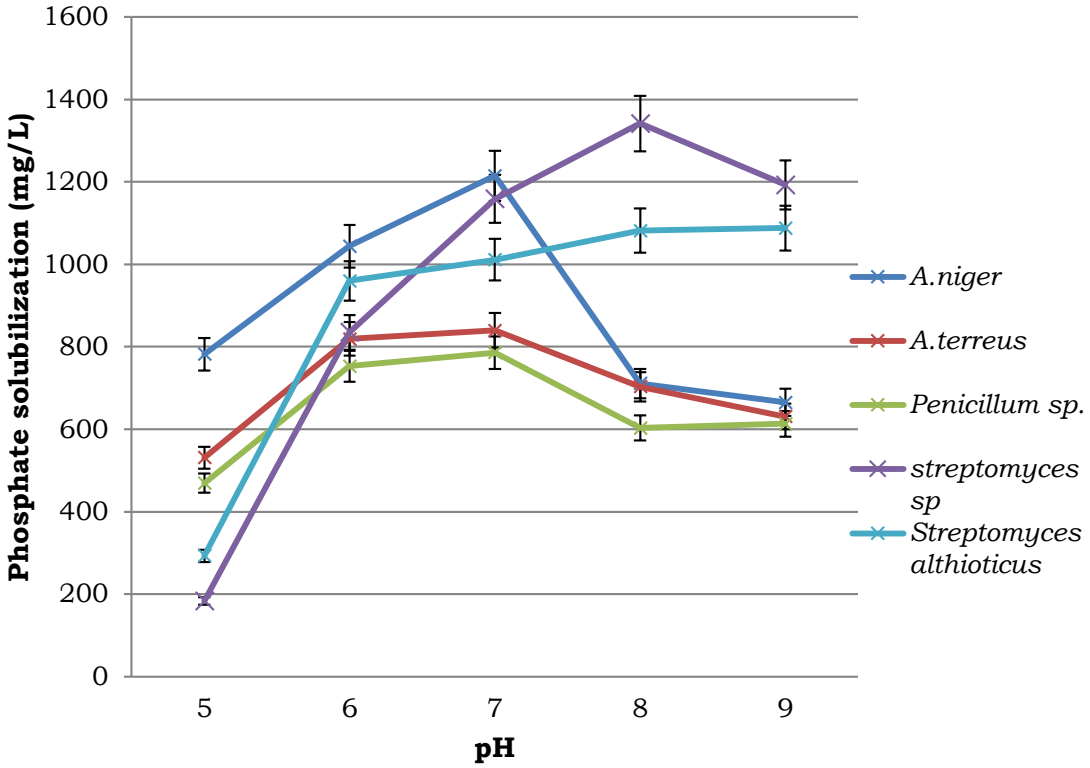

Fig.3. Effect of pH on solubilization of $Ca_3 (Po_4)_2$ by selected strains *A.niger* US11, *A.terreus* US3, *Penicillium* sp.US10, *Streptomyces* sp.US16, *S. althioticus* US5

Table-1 Effect of different concentration of TCP on solubilization potential of *A. niger* US11, *A. terreus* US3, *Penicillium* sp. US10, *Streptomyces* sp. US16, *S. althioticus* US5

Strain name	Concentration of TCP(mg/L)	Phosphate solubilization (mg/L)						
		24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours
Aspergillus niger US11	2500	130±1.3	370±6.8	680±6.9	826±2.2	970±4.1	1025±1.8	933±3.1
	5000	206±5.2	571±2.8	911±4.2	955±1.4	1022±1.4	1215±3.1	985±2.2
	7500	216±5.7	490±0.5	985±2.6	1105±1.5	1170±5.2	1353±2.1	996±5.4
	10000	198±3.3	502±2.3	898±0.7	905±2.0	982±1.4	1301±4.2	895±5.4
Aspergillus terreus US3	2500	118±3.0	182±0.3	592±1.4	800±00	522±2.1	365±2.5	295±8.5
	5000	144±3.1	195±5.8	530±2.5	842±1.5	539±2.3	289±4.2	268±2.8
	7500	265±3.8	510±3.0	823±4.2	1185±3.1	989±4.2	435±8.9	405±2.8
	10000	195±2.3	469±4.2	819±1.8	1071±7.1	830±2.9	759±5.8	620±7.1
Penicillium sp. US10	2500	80±2.3	110±6.6	177±6.7	595±2.1	707±1.4	416±1.7	401±5.7
	5000	85±2.5	114±3.5	193±3.1	765±5.7	739±2.4	408±5.5	396±2.8
	7500	78±2.7	102±1.2	213±2.3	715±1.0	696±1.0	329±2.5	328±0.0
	10000	79±4.7	112±2.0	203±5.8	699±0.0	656±5.4	385±1.0	370±5.9
Streptomyces sp. *US16*	2500	405±2.1	711±5.7	818±5.6	672±5.5	608±4.3	522±1.9	419±2.5
	5000	584±2.2	917±1.4	1192±1.4	958±5.7	680±3.1	660±00	582±5.3
	7500	295±4.5	600±00	621±4.2	553±5.0	543±3.2	432±5.2	403±5.5
	10000	199±0.0	453±00	571±4.2	517±1.9	468±5.2	495±00	395±00
Streptomyces althioticus US5	2500	439±0.0	771±1.7	992±1.7	911±1.4	752±4.1	671±5.4	690±1.0
	5000	519±1.3	1061±2.2	1122±1.4	968±5.8	862±1.4	857±1.4	843±1.4
	7500	400±0.0	830±00	935±7.1	842±2.5	717±1.0	696±5.2	600±1.0
	10000	325±1.5	685±1.1	818±2.2	759±5.8	630±4.5	539±0.5	541±0.1

TABLE:2 VARIATION IN PH OF CULTURE FILTRATES DURING DIFFERENT INCUBATION TIME

	pH of culture filtrates				
Strains → Time (Hours) ↓	***A. niger* US11**	***A. terreus* US3**	***Penicillium* sp. US10**	***Streptomyces* sp.US16**	***Streptomyces althioticus* US5**
0	7.0	7.0	7.0	7.0	7.0
24	6.0	5.6	6.5	7.8	7.5
48	5.3	5.1	5.0	7.9	7.9
72	4.3	4.2	4.5	8.2	8.7
96	2.5	3.0	3.3	8.9	8.4
120	1.5	3.2	3.9	8.0	8.0
144	1.5	3.5	4.5	8.1	8.5
168	2.8	4.1	4.5	7.9	7.9

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