

## **CANDIDATE'S DECLARATION**

I hereby declare that the work which is being presented in the dissertation entitled, " Isolation and screening of phosphate bacteria showing alkaline phosphatase activity" in the partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN BIOTECHNOLOGY, is an authentic record of my own work during a period of five months from January 2003 to May 2003, under the supervision of Dr. Sunil Khanna, HOD, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

PLACE

DATE

( Vinay Kumar)

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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## ACKNOWLEDGEMENT

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I have found this rare opportunity to evince a word of thanks to all those who played a key role in the successful completion of my project. I wish to express my sincere and deep sense of reverence for Dr. Sunil Khanna, my esteemed advisor and Head of Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology (Deemed University), Patiala, who with his able guidance, endless patience, altruistic help, continuous encouragement, constructive criticism and affectionate attitude, made me work on the problem. I am highly obliged to him for the cordial atmosphere in which he guided me throughout the period of investigation.

My sincere thanks to Dr. N. Das, Dr. D. Goyal, Dr. M.S. Reddy, Dr. P. Khandelwal & Dr. S. Saxena for their cooperation, guidance during experimental work, frank opinion and nice behavior.

My gratitude is due to Dr. A. Ganguly and Mr. A. Roy Choudhary for their valuable suggestions, help and moral support.

I also feel very much obliged Mr. A. Aggarwal and Mr. O. P. Yadav for their constant cooperation and help.

Words are insufficient to express my gratefulness and indebtedness to my friends, Mohit, Machiavelli, , Vidya, Ritu & Kamal for giving me the moral support, love, understanding and dependence during my critical times. I am deeply beholden by warmth and cooperation given by Jagdish and Rajeev who stood by my tough times, crucial and happy moments. I also thank to Rajesh, Naib & Sandeep for joyful atmosphere.

Finally, most of all, I thank my family members for their unconditional love, encouragement and support to complete my project work.

I also thank all those who could not find a separate name but have helped directly and indirectly.

(Vinay kumar)

DATE May, 2003

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## ABBREVIATIONS AND SYMBOLS

The abbreviations for chemicals and symbols follow either the tentative rules of IUPAC– IUB Commission on Biochemical Nomenclature Biochem. J. (1966) 101, 1-7 or the instructions to Authors by the Biochemical Journal (Biochem. J. (1973) 131: 1-20).

°C	degree centigrade
cm	centimeter
g	gram
hr	hours
μl	microliter
μ	micro
ml	milliliter
μg	microgram
mg	milligram
mm	millimeter
M	molarity
mM	millimolar
mMoles	millimoles
min	minute
N	normality
OD	optical density
%	per cent
temp	temperature
TCP	tri calcium phosphate
PM	Pikovskaya media

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# INTRODUCTION

## INTRODUCTION

India is basically an agricultural country. The economic stability of India is dependent on the agricultural yield. Modern agriculture is evolving with much greater emphasis on the efficiency of the production process than in the past & great deal of research has been carried in the last 50 years by agricultural scientists has its major thrust on increasing production and productivity. The process has gathered momentum with the development of high yielding varieties of crop plant in response to the challenge of feeding the rapidly increasing human populations in most developing countries. The new technology has already made an impact although the crop yields in most developing countries continue to be low relative to the genetic potential, which has been created. A major factor contributing to this gap is the heavy dependence on costly non-renewable resources of energy such as chemical fertilizers.

Most developing countries do not have the industrial development, which helps to subsidize high input agriculture in developed countries. For this reason, and as a result of major scientific advances in the field of microbiology and molecular genetics in recent years, scientists are exploring new ways of meeting the nutrient needs of crop plant aiming at high productivity. Soil microorganisms like bacteria; cyanobacteria and fungi have a particularly important role in the exploration of these new approaches.

Phosphorous is an important plant nutrient, next only to nitrogen and classed along with nitrogen and potassium as a major plant nutrient element. Pierre (1938) referred to it as the `Master Key` element in crop production. It is associated with several vital functions and is responsible for several characteristics of plant growth such as utilization of sugars and starch, photosynthesis, nucleus formation and cell division, fat and albumin formation, cell organization and the transfer of heredity (Arnon 1936; McVicker etal.1963).

## **ROLE OF PHOSPHATE SOLUBILIZING MICROORGANISMS IN CROP PRODUCTIVITY**

In India phosphorus content of most soils is quite low (Chakravati 1964, Raheja 1966), so the application of phosphatic fertilizer in available form is essential for better crop yield. It is well known that more than 2/3<sup>rd</sup> of phosphatic fertilizer is rendered unavailable within a very short period of its application due to fixation in the soil complex (Hemwall 1957, Langguth et al. 1957, Wild 1970, Mandal and Khan 1972).

It has been established that there are specific groups of soil micro-organisms which increase the availability of phosphates to plants not only by mineralizing organic phosphate compounds but also by rendering inorganic phosphorus compounds more available to them (Gerretses 1948, Mishustin and Naumova 1962 a & b, Menkina 1963, Sundara Rao 1965, Taha et al. 1969, Tardieux-roche and Tardieux 1970, Gaur 1972, Jackson et al. 1972, Bardiya and Gaur 1974, Arora and Gaur 1979). Considerable success was earlier claimed, particularly by Russian workers, in increased yields and quality of crops by inoculating seeds with pure and efficient strains of *Bacillus megaterium* var. *phosphaticum* commonly called "Phosphobacterin" mineralizing orthophosphate (Kudashev 1956, Klashnikov 1957, Mishustin and Naumova 1962 a & b, Menkina 1963, Mishustin 1967). Microbial solubilization of inorganic & organic phosphatic compounds has been extensively studied under Indian conditions (Sundara Rao *et al.* 1963, Gaur 1972, Gaur and Oswal 1972, Gaur and Singh 1982). Therefore one of the approaches would be to increase the number and activity of efficient PSM in the root zone of plants by use of microbial inoculants for increasing phosphorus availability to the plants from the soil as well as added phosphate.

It is estimated that India alone has about 140 million tonnes of rock phosphate deposits, most, of which are low grade and contain impurities. Only high-grade rock phosphates, free from impurities, are utilized for the manufacture of phosphatic fertilizers. Direct use of even low-grade rock phosphate as fertilizer is feasible in neutral to alkaline soils if PSM are used as inoculants.

## POOLS OF PHOSPHORUS IN SOIL AND FLOWS BETWEEN POOLS

In the following scheme a number of pools are shown in which P can be found, both in the solid phase of the soil and within the soil solution. In the solid phase a distinction is made between organic (left) and inorganic forms (right), in the soil solution between ortho-P and dispersed colloidal P. The last fraction can be either adsorbed on inorganic colloids (clay particles, Fe/Al-hydroxides), or is bound to dissolved organic matter (DOC).

SOLID PHASE	SOLUTION	SOLID PHASE
organic form		inorganic form
living biota		precipitated
fresh organic material	ortho-p	adsorbed
stable organic matter		adsorbed {in aggregates}

## DIFFERENT FORMS OF PHOSPHOROUS

- *Inorganic forms of phosphorous*

Large quantities of phosphorus in inorganic forms occur in minerals as insoluble calcium, iron or aluminium phosphates. The native soil phosphorus probably originated from the dissolution of rock containing mineral apatite,  $\text{Ca}_{10}(\text{PO}_4, \text{CO}_3)_6(\text{F}, \text{OH}, \text{Cl})_2$ . This mineral may be in the form of carbonato-, chloro- or hydroxyapatite or in combination of these as the formula shows. In soil, phosphorus is found as fluorapatite, hydroxyapatite or chlorapatite, as iron or phosphate or in combination with the clay fraction. The calcium phosphates are dominant in neutral to alkaline soils whereas iron and aluminium phosphates occur in acidic soils. Organic phosphorus

Vegetation and decaying plant residues entering the soil are the main sources

of organic phosphorus compounds. Crop residues contain 0.05 to 0.5 percent phosphorus. Organic forms comprise phytins, phospholipids, nucleic acids, phosphorylated sugars and coenzymes.

In contrast to the cases of nitrate ( $\text{NO}_3^-$ ) sulphate ( $\text{SO}_4^-$ ), plants do not reduce phosphate and this ion enters into organic combination mostly unchanged. Therefore, the phosphorus in phosphatic compounds is found as phosphate. Phytin is a calcium magnesium salt of phytic acid (inositol phosphate). Inositol phosphate may have one to six phosphorus atoms per inositol unit. Phospholipids include lecithin and cephalin; the phosphate is esterified with a nitrogenous base. Lecithin is made up of glycerol, fatty acids, phosphate and choline. Nucleic acids, ribose nucleic acid (RNA) and deoxyribose nucleic (DNA) consist of a number of purine and pyrimidine bases, pentose and phosphate. The bulk of phosphorus in the bacterial cell is found in RNA, and DNA contributes only from 2 to 10 per cent of total P. In soils, 5 to 85 per cent of total phosphorus is organic. Surface vegetation, microbial protoplasm or metabolic products of microflora contribute the organic compounds making up the humus fraction. There are several more reports on the factors controlling the liberation of phosphate by decomposition of organic matter than on its immobilization during the humification process. Important are drying and wetting and high temperature. Probably humus compounds, due to drying and re-wetting processes, become more to microbial attack and augmented microbial activity caused by favourable temperature. In the great majority of arable soils, it is of minor importance.

**The main objective of this work are:**

1. Development of bacterial consortium capable of phosphate solubilization and isolation of efficient bacterial isolates.
2. Estimation of the phosphatase activity of the selected isolates at alkaline pH.

# REVIEW OF LITERATURE

## REVIEW OF LITERATURE

### Phosphorus solubilizing micro-organisms (PSM)

PSM include different groups of microorganisms such as bacteria and fungi, which convert inorganic phosphatic compounds into soluble form. Such bacteria and fungi can grow in media where  $\text{Ca}_3(\text{PO}_4)_2$ ,  $\text{FePO}_4$ ,  $\text{Al}(\text{PO})_4$ , apatite, bone meal, rock phosphate or similar insoluble phosphate compounds are the sole source of phosphate compounds are the sole source of phosphate. Such organisms not only assimilate phosphorus but also cause a large portion of soluble phosphate to be released in quantities in excess of their own requirements.

**Table No.1: BACTERIA, FUNGI AND ACTINOMYCETES SHOWING PHOSPHATE SOLUBILIZING ACTIVITY (Subba Rao, 1986).**

Bacteria	Fungi and Actinomycetes
<i>Bacillus sp.</i> , <i>B. circulans</i> , <i>B. mycooides</i> , <i>B. mesentericus</i> , <i>B. megaterium</i> var. <i>phosphaticum</i> , <i>B. polymyxa</i> , <i>B. subtilis</i> , <i>B. pulvifaciens</i> , <i>B. coagulans</i>	<i>Aspergillus sp.</i> , <i>A. flavus</i> , <i>A.</i> <i>awamorii</i> , <i>A. niger</i> , <i>A. terreus</i> , <i>A.</i> <i>fumigatus</i> , <i>A. nidulans</i>
<i>Pseudomonas sp.</i> , <i>P. calcis</i> , <i>P.</i> <i>liquifaciens</i> , <i>P. rathonis</i> , <i>P. fluorescens</i> , <i>P. putida</i> , <i>P. striata</i>	<i>Tricoderma viride</i>
<i>Flavobacterium sp.</i>	<i>Fusarium sp.</i> , <i>F. oxysporum</i>
<i>Brevibacterium sp.</i>	<i>Penicillium sp.</i> , <i>P. lilicinum</i> , <i>P.</i> <i>digitatum</i>
<i>Alcaligenes sp.</i>	<i>Curvularia lunata</i>
<i>Achromobacter sp.</i>	<i>Humicola</i>
<i>Aerobacter aerogenes</i>	<i>Sclerotium rolfsii</i>
<i>Xanthomonas sp.</i>	<i>Alternaria tenuis</i>
<i>Erwinia sp.</i>	<i>Phythium</i>
<i>Serratia sp.</i>	<i>Acrotheceium</i>
<i>Micrococcus sp.</i>	<i>Phoma</i>
<i>Escherichia freundii</i> , <i>E. intermedia</i>	<i>Mortierella</i>
<i>Nitrosomonas</i>	<i>Paecilomyces</i>
<i>Nitrobacter</i>	<i>Cladosporium</i>
<i>Ferrobacillus ferrooxidans</i>	<i>Rhodotorula</i> , <i>Schwanniomyces</i> <i>occidentalis</i>
<i>Thiobacillus thiooxidans</i> , <i>ferrooxidans</i>	T. <i>Cunninghamella</i> , <i>Candida sp.</i>
<i>Rhizobium meliloti</i>	<i>Actinomycetes</i>

Aluminum, magnesium, manganese and other phosphates are also acted upon. Stalstorm (1903) demonstrated solubilization of tricalcium phosphate by bacteria from milk and soil infusion in liquid medium. Sackett et al. (1908) showed the solubilization of tricalcium phosphate, bone meal and rock phosphate by soil bacteria in agar and liquid medium plates. Bromofield (1959) reported dissolution of rock phosphate by fungi mainly by production of organic acids in medium. Konig (1961) showed that organic acids liberated by fungi dissolved phosphate ions by the fungal mycelia, which were ultimately released by autolysis of the organisms. Ahmad and Jha (1968) published results regarding the solubilisation of hydroxyapatite and rock phosphate by Gram +ve and Gram -ve rods, cocci-shaped bacteria and fungi (*Aspergillus*, *Penicillium* and *Rhizopus*) and species of *Nocardia* and *Micromonospora*. Sethi and Subba Rao (1968) studied the ability of fungi, particularly species of *Acrothecium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Phoma* and *Rhizoctonia* to mineralize calcium phytate. Among them, *Aspergillus* and *Penicillium* were most active in mineralize calcium phytate at varying pH levels to phytase. Bardiya and Gaur (1972) studied solubilisation of types of rock phosphates by different groups of bacteria and fungi. They reported that fungi solubilised phosphate more than bacteria. Nair and Rao (1977) reported isolates of *Pseudomonas sp.* and *Aspergillus sp.* in rhizospheres of coconut. Singh et.al. (1984) Studied solubilisation of rock phosphate from different sites and found that *Aspergillus awamori* was a better solubilised than *Bacillus polymyxa* and *Pseudomonas sp.* . Nahas et al (1990) reported fluorapatite solubilisation by *Aspergillus niger* in Vinasse medium. Cunningham and Kuiack (1992) reported the production of citric acid and oxalic acid along with solubilisation of calcium phosphate by *Penicillium bilaii*. Altomare, et. al. (1999) reported solubilisation of phosphates by plant growth promoting fungus *Trichoderma harzianum* .

Pilovskaya (1948) suggested a medium containing tricalcium phosphate with glucose, yeast extract and other salts for growth and of phosphorus solubilizing microorganisms. This medium has been widely used for isolation, enumeration and maintenance or phosphate dissolving

microorganisms. Addition of insoluble phosphatic like di- or tricalcium phosphate as fine powder in the medium has also been suggested (Johnston 1951, Bunt and Rovira 1955, Louw and Webley 1958). Both the methods (such as fresh precipitation of dicalcium phosphate or hydroxapatite, or incorporation of finely insoluble phosphate compounds in the medium) are followed for the isolation of phosphate dissolving microorganisms. Gaur (1972) and Bardiya and Gaur (1974) have suggested that the isolation of efficient rock phosphate solubilizers is an appropriate approach because they also show capability of dissolving different forms of insoluble phosphates (Gaur and Gaur, 1983). Serial dilution of soil and rhizosphere soil samples are plated on agar medium containing suspended insoluble phosphate compounds. A 1g sample is suspended in a known volume of sterile water and serial of the suspension are made in sterile water blanks. The plates are incubated up to two weeks at  $28\pm 2^{\circ}$  C. The colonies showing halo zones (Plate 1) around them are picked up and transferred to slants (Bardiya and Gaur 1974, Arora and Gaur 1979, Gaur unpublished). Factors affecting solubilization of insoluble phosphates appropriate carbonaceous substrates, nutrients and proper conditions are important parameters for maximum solubilization.

### **Carbon sources**

Since these microorganisms are heterotrophs and solubilize insoluble phosphates by secreting organic acids, the role of carbon sources in this context is well understood. However, the researches finding in this context are not many. Rose (1957) showed that glucose or xylose was best source of energy for fungi in liquid medium, whereas Katznelson and Bose (1959) and Sobieszcsanki (1961) reported that either yeast extract or soil extract was essential for the proper growth of phosphorus solubilize organisms in liquid or solid medium. The solubilization of rock phosphate using diverse types of carbon sources was studied by Bardiya and Gaur (unpublished). It was observed that rock phosphate solubilize bacteria, yeast and fungi utilized a variety of carbon compounds as energy sources, but the amount of phosphate solubilized varied significantly with different sources of energy. Glucose,

sources, galactose and arabinose were found to be energy and carbon sources for *Pseudomonas striata* and were effective in the order listed. *Schwanniomyces occidentalis* utilized only a few carbon sources such as source, fructose and glucose. *Aspergillus awamori* preferred sucrose, mannitol, glucose, arabinose, xylose and galactose, in this order. *Penicillium digitatum* solubilized the maximum P<sub>2</sub>O<sub>5</sub> in the presence of glucose, followed by sucrose, mannitol, arabinose, fructose, xylose and galactose. Most of them could be used efficiently, but the minimum solubilization was observed in the presence of galactose. Similarly, the solubilization by *A. awamori* was minimum with galactose as the carbon source. The results showed that, generally, glucose and sucrose were the best sources for strains of *P. striata* or *S. occidentalis*. *A. awamori* could use different carbon sources but its best sources were mannitol, glucose, fructose and sucrose. *P. digitatum* could favourably use glucose, sucrose and mannitol, in this order. Gaur Gaiand (1983) reported that during 15 days of incubation in liquid medium, maximum rock phosphate solubilization by *A. awamori* was obtained with sucrose or mannitol as compared to glucose. It is evident that, for mass production, sucrose, a cheaper substitute for , can be used for Pikovskaya's medium.

### **Nitrogen sources**

Microorganisms for the synthesis of amino acids, proteins and purine and pyrimidine nitrogenous bases need nitrogen. Nitrogen may be taken up by micro-organisms either in the form of salts of ammonia, nitrite, nitrate or in amino form, depending upon the enzyme present in the system. The strains of *P. striata* utilized different forms of nitrogen, except for nitrites. *S. occidentalis* could utilize only ammonium or amino-nitrogen forms. They could not use nitrates, indicating that these organisms may be lacking in enzymes assimilatory for nitrate reduction. However, ammonium sulphate was the best source for both the bacteria and the yeast examined. The fungi could grow and solubilize good amounts of rock phosphate using different nitrogen forms, but the best source was ammonium nitrate followed by asparagine for *A. awamori*, and sodium nitrite followed by potassium nitrate and asparagine for *P. digitatum*.

## Hydrogen ion concentration

The pH of the medium is known to affect the growth and activity of microorganisms. The optimum pH range for maximum rock phosphate solubilization was studied. The maintained pH value changed appreciably due to sterilization of the medium. Such changes in the pH values of the medium due to sterilization have been observed by several workers (Goswami and Sen 1962, Bajpai 1965, Taha et al. 1969). The selected efficient micro-fungi were capable of solubilizing rock phosphate over a wide range of pH from 5.0 to 7.8 (before sterilization pH values were 4 to 9.6). The bacteria could solubilize maximum phosphate at around pH 6 whereas the activity of the fungi and the yeast was best in the pH range of 5-6, particularly in case of *Aspergillus awamori*. Fungal activity was greatly reduced at pH 7 to 8. Bajpai and Sundara Rao pH 4.0 for fungi for maximum solubilization of tricalcium phosphate in Pikovskaya medium, the best pH was around 6 for fungi (*A. awamori* and *P. digitatum*) and 7 to 8 for the bacteria (*P. striata* and *B. polymyxa*). Temperature is a vital factor for the growth and activity of micro organisms. A range between 20 and 30° C was found suitable for rock phosphate solubilization by strains of *Pseudomonas striata* although 25-30° C was more favourable. For *Aspergillus awamori* and *Penicillium digitatum*, 30° C was the optimum. The activity of these microorganisms was drastically reduced at higher temperatures (35 and 40° C). At higher temperature, the culture medium was found a little less acidic. There is need to isolate active bacteria and fungi which can grow at higher temperature, at least up to 45° C. Wani *et al.* (1979) reported that 30° C was optimum for tricalcium phosphate solubilization by *P. striata*, *A. awamori* and *P. digitatum*, whereas 35-40° C favoured phosphate solubilization by *Bacillus polymyxa*.

## Aeration

A significant increase in rock phosphate solubilization was observed in shake culture as compared to static culture with three different strains of *P. striata* and *Aspergillus awamori*. These findings are supported by other workers (Sinha 1961, Ahmad and Jha 1968). On the contrary, *S. occidentalis* and *P. digitatum* solubilized less rock phosphate under shake culture. Ahmad and

Jha (1968) also observed less solubilization by a *Rizopus* sp. under shake culture. Incubation period Several investigations have shown that, under cultural conditions, phosphate solubilization progressively increases up to 10-15 days (Goswami and Sen 1962, Wani *et al.* 1979, Gaur and Sachar 1980, Gaur and Gaiind 1983). Ortuno *et al.* (1977/78) reported that tricalcium phosphate solubilization started after 24 hours of incubation and reached a maximum on the 7<sup>th</sup> and 15<sup>th</sup> days with *Aspergillus niger* and *Pseudomonas fluorescens* respectively. The findings presented in will illustrate the observation (Bardiya and Gaur, unpublished).

### **Humic substances**

*Bacillus megaterium* var. *phosphaticum* solubilized increased amounts of phosphate from insoluble tricalcium phosphate in the presence of sodium humate and fulvic acid. In fact, it was noted that the effect of these substances was better on phosphorus solubilization by this bacterium than on its growth. The amounts of phosphate solubilized are in proportion to the quantities of sodium humate and fulvic acid added. Fulvic acid proved more effective than sodium humate in increasing the efficiency of phosphorus solubilization by *B. megaterium*. The result also showed that humic and fulvic acids themselves react with the insoluble  $\text{Ca}_3(\text{PO}_4)_2$  and release soluble phosphate although the amounts solubilized are comparatively smaller than that released by the bacterium or in combination with it. Increasing quantities of these substances liberated increasing amounts of soluble phosphate. Fulvic acid released more phosphate than sodium humate. Humic substances act as strong complex forming and chelating agents, better than or comparable to synthetic chelating agents (Titova 1962, Aso and Sakai 1963, Gaur 1969, Pareek and Gaur 1973). The chelation property of humic substances is of great use in increasing the efficiency of phosphatic fertilizers, which are rendered insoluble through fixation mechanisms.

## **MECHANISMS FOR SOLUBLISING PHOSPHOROUS**

### **Production of organic acids**

A fall in pH during the growth of phosphate solubilizing micro-organisms in liquid medium containing insoluble phosphates has often been reported (Gerretsen 1948, Muromtsev 1958, Sperber, 1958a, b, Swaby and Sperber 1958a, Bromfield 1959, Louw and Webley 1959, Moreau 1959, Sobieszczanski 1961, Tardieux-Roche 1966, Chhonkar and Subba Rao 1967, Ahmad and Jha 1968, Sethi and Subba Rao 1968, Sunna rao and Bajpai 1965, Taha et al. 1969, Gaur 1972, Bardiya and Gaur 1972,1974, Arora and Gaur 1979, Kundu and Gaur 1981, Sattar and Gaur 1984). The studies have shown that during the solubilization of rock phosphate and tricalcium, iron and aluminium phosphate by fungi, the pH of the culture broth was lowered from 7 to 3 (Gaur and Sachar 1980, Gaur and Gaur 1983, Venkateshwarlu *et al.* 1984). Phosphate dissolving micro-organisms were found to produce monocarboxylic acid (acetic, formic); monocarboxylic hydroxy (lactic, gluconic, glycolic); monocarboxylic keto (2-keto gluconic); dicarboxylic (oxalic, succinic); dicarboxylic hydroxy (malic, maleic) and tricarboxylic hydroxy (citric) acids in liquid media from simple carbohydrates. Bajpai and Sundara Rao (1971c) reported that nonvolatile acids such as citric and lactic acids were produced by *Bacillus megaterium*, *circulans* and *Escherichia freundii* from glucose in Pikovskaya medium. Mishustin *et al.* (1972) studied, in a model experiment, solubilization of tricalcium phosphate in liquid medium by citric, gluconic, succinic and oxalic acids which depended on pH and formation of soluble Ca-complexes.

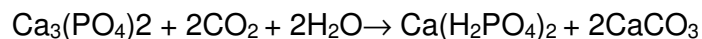
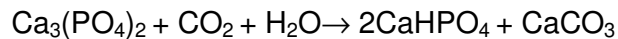
### **CHEMICAL EXPLANATION**

The role of organic acids in dissolving mineral phosphates and phosphorylated minerals can be attributed to the lowering of pH, which helps in the formation of stable complexes with such cations as  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Fe}^{++}$  and  $\text{Al}^{+++}$ . These complexes are more stable than the original inorganic phosphate compounds. Similar reactions are undoubtedly involved in preventing the fixation of chemical phosphate fertilizer or phosphates formed

in situ by the weathering of minerals (Stevenson et al. 1949, Struthers and Sieling 1950, Dalton *et al.* 1952, Bradley and Sieling 1953, Mortenson 1963).

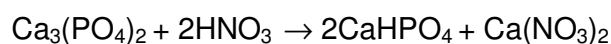
### **Effect of Carbon dioxide**

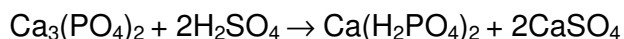
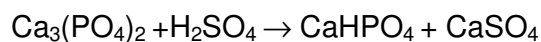
The early researchers in this field believed that carbon dioxide produced by plant roots and micro flora was responsible for solubilization of inorganic phosphates in soils (Stoklasa and Ernest 1908). Later, it was found that carbon dioxide increased the availability and absorption of phosphates by plants in calcareous soils (Truog 1927, McGeorge 1938). Starkey (1931) emphasized the importance of the rhizosphere flora to growing plants and suggested that carbon dioxide production in soil might affect the solubilization of plant nutrients. Carbon dioxide produced by microorganisms in the rhizosphere seems to be an effective mechanism in increasing the availability of phosphate and its uptake by crop plants. This view has also been shared by others (Gerretsen 1948, Pierre 1948, Waksman 1952 and Norman 1953). Solubilization of inorganic phosphate by bacteria and fungi in liquid medium due to carbon dioxide production was also reported by Moreau (1959). The reaction may be summarized as follows.



### **Inorganic acids**

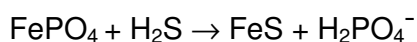
Nitric or sulphuric acid is produced due to oxidation of nitrogenous compounds or inorganic compounds of sulphur by nitrifying bacteria and *Thiobacillus* spp. respectively, which react with chemical phosphates, converting them into soluble form. The possible chemical reactions are as follows.





### **H<sub>2</sub>S Reaction**

Although acid solubilization of phosphate is more common another mechanism under anaerobic conditions may be involved for ferric phosphate solubilization. Hydrogen sulphide may be formed by reduction of sulphur-containing amino acids by several heterotrophic microorganisms or from sulphate by sulphate reducing bacteria of the genus *Desulfovibrio*. Hydrogen sulphide thus formed reduced ferric phosphate to black ferrous sulphide with the release of available phosphate as shown in the following reacting:



Swaby and Sperber (1958) reported that H<sub>2</sub>S produced under anaerobic conditions as in waterlogged soils and treatment with sulphur increased P<sub>2</sub>O<sub>5</sub> availability. In flooded soil, the iron in insoluble ferric phosphate may be reduced, resulting in the formation of soluble iron with simultaneous release of phosphorus into solution (Patrick *et al.* 1973).

### **Alkalinity**

Another mechanism of phosphate solubilization is the development of alkalinity in the liquid medium during the growth of phosphate solubilizing micro-organisms. Alkali production in the medium by some tricalcium phosphate dissolving bacterial and fungal isolates has been reported by a few workers (Muromtsev 1958, Bromfield 1959, Goswami and Sen 1962, Tardieux-Roche 1966, Chhonkar and Subba Rao 1967, Ahmad and Jha 1968).

### **Mineralization of organic phosphorus**

Soil organic matter or plant and animal residues contain large quantities of organic phosphorus which cannot be utilized by plants without their microbial conversion to inorganic form. Bacteria, fungi and actinomycetes by their

enzymatic action make the bound organic phosphates available to plants. In contrast to nitrogen and sulphur, the plant does not reduce phosphate. This ion enters into organic combination without any appreciable alteration. Warm temperatures the thermophilic range being more favourable than the mesophilic range also favour mineralization. The rate of mineralization is augmented by a shift in the pH from acidity to neutrality. The rate of mineralization is generally correlated to the quantity of the substrate. Hence, soils rich in organic phosphates will be most active. The mineralization of organic phosphorus is not inhibited by inorganic phosphates (Daughtrey *et al.* 1973).

### **Mechanism of microbial mineralization**

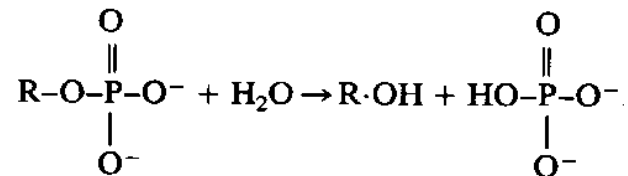
Organic phosphorus consists mainly of phytin, nucleic acids and lecithin. The enzymes, which cleave phosphorus from organic substrates, are known as phosphatases. Phytins, nucleic acids and lecithins are mineralized by the enzymes phytase, nuclease and lecithinase respectively under favourable environmental conditions. A single phosphatase may act on different substrates, R-group being the variable. Thus, one enzyme may act on cleavage of ethyl phosphate, glycerophosphate and phenyl phosphate. On the other hand, molecules with two R-groups (di-esters) may require other enzymes for their cleavage.

Phospholipases and nucleases have di-esters as their substrates. The enzymes catalyzing the hydrolysis of the monoesters act at either acidic or alkaline pH and thus are designated as acid or alkaline phosphatase.

### **PHOSPHATASE ENZYME**

Phosphatases are broad enzymes that catalyze the hydrolysis of both esters and anhydrides of  $H_3PO_4$ . These enzymes are responsible for soil organic phosphorus mineralization and the release of inorganic phosphorus needed by microorganisms and plants. Phosphatases are collective name for enzymes (extracellular) that cleave phosphate from organic compound (e.g. phospholipids, nucleic acids). The enzymes are classified as acid and alkaline

phosphatases because their maximum activities occur at low (pH 6.5) and high (pH 11) pH ranges. Both microorganisms and higher plants produce acid phosphatases, but mainly microorganisms produce alkaline phosphatases. Alkaline phosphatases refer to the optima for enzymes. They catalyze the following reaction:



### Genetic of phosphatase

The expression of phosphatase gene is under the control of *pho* regulon and the expression of the *Pho* regulon genes requires the two-component regulatory proteins *PhoP* and *PhoR* and enables cells to use limiting phosphate resources more efficiently or to make accessible alternative phosphate sources (Antelmann *et al.*, 2000). These *Pho* regulon genes include:

- ♣ The *phoA* and *phoB* (alkaline phosphatases) (which account for 98% of total APase activity) (Bookstein *et al.*, 1990)
- ♣ The *pstS* (inorganic phosphate transport system)
- ♣ The *tuaA* (teichuronic acid biosynthesis)
- ♣ A gene encoding an APase-alkaline phosphodiesterase (APDase), *phoD*, which has a putative role in cell wall teichoic acid turnover. (Eder *et al.*, 1998)
- ♣ The high-affinity phosphate transport operon, *pstSACB1B2* (Mori *et al.*, 1999)
- ♣ The *tuaABCDEFGH* operon, which is responsible for the synthesis of teichuronic acid, which replaces the teichoic acid in the cell walls of phosphate-starved cells. (Liu *et al.*, 1998)

- ♣ The teichoic acid biosynthesis operons, *tagAB* and *tagDEF*, whose transcription was shown to be repressed by PhoP and PhoR (Liu *et al.*, 1998)
- ♣ the *phoPR* operon, encoding PhoP and PhoR (Seki *et al.*, 1987; Seki *et al.*, 1988).

The phosphatase is induced by the response of phosphate starvation. Under phosphate starvation conditions, several genes in *B. subtilis*, including *phoA* and *phoB* (alkaline phosphatases), *pstS* (inorganic phosphate transport system), *tuaA* (teichuronic acid biosynthesis), are activated by the two-component (PhoP-PhoR) regulatory system response regulator and histidine kinase.

All *pho*-regulated promoters require a minimum of four consensus PhoP binding sequence repeats (TT(A/T/C)A(C/T)A) for activation (Robichon *et al.*, 2000) which repeated at intervals of 11 bp and separated by approximately 5 bp in the promoter regions of *phoA*, *phoB*, *phoD*, *pstS*, *tuaA*, and *tagAD* (Eder *et al.*, 1999; Liu *et al.*, 1997; Liu *et al.*, 1998; Liu *et al.*, 1998; Qi *et al.*, 1998). And all these four repeats are required for PhoP-P binding and transcriptional activation; therefore, this conserved sequence arrangement is termed the core binding region (Qi *et al.*, 1998). It has been shown that a dimer of PhoP-P is able to bind two consensus repeats in a stable fashion (Eder *et al.*, 1999). The stronger Pho regulon *phoA* and *pstS* promoters contain secondary PhoP binding sites which consist of fewer than four TT(A/T/C)ACA-like repeats within the coding region and which are required for promoter activation (Liu *et al.*, 1998). The *phoD* promoter was characterized as the strongest Pho regulon promoter and contains the core binding region and a 5' secondary binding region, which is important for coordinated PhoP binding to the core-binding region. It was hypothesized that PhoP binding to the core and secondary binding regions results in DNA loop formation to activate transcription from the stronger Pho regulon promoters (Eder *et al.*, 1999).

The Pho regulon genes are well characterized (Eder *et al.*, 1998; Hulett *et al.*, 1991; Liu *et al.*, 1998; Liu *et al.*, 1998; Seki *et al.*, 1987; Seki *et al.*, 1988) and

most of these were identified on the cytoplasmic as well as the extracellular proteome map. On the cytoplasmic proteome map, three components of the high-affinity phosphate transport system could be identified. These included, in addition to the previously identified phosphate binding protein PstS, the ATP binding proteins PstB1 and PstB2. PstS was the most strongly induced protein (Eymann *et al.*, 1998). The low-level induction of PstS observed in the *phoR* mutant is consistent with the transcriptional data for the *pstS* promoter (Qi *et al.*, 1997). Because the *pstS* promoter could be activated only by PhoP-P, it was proposed that PhoP might be phosphorylated by other histidine kinases in the *phoR* mutant (Qi *et al.*, 1998). The stronger induction of *pstS* than of other Pho regulon genes was found to be due to the secondary PhoP binding region in addition to the core binding region, which is located within the coding region of *pstS* (Liu *et al.*, 1998).

The *tua* operon encodes proteins involved in the biosynthesis of teichuronic acids, which replace phosphate-containing teichoic acids (Liu *et al.*, 1998). APase specific activity increases 500-fold after transition into the stationary phase under low-phosphate conditions (Antelmann *et al.*, 2000). A third APase structural gene, *phoD*, encodes an enzyme with both APase and phosphodiesterase activities and also belongs to the Pho regulon (Eder *et al.*, 1998). To a minor extent, the APase PhoB and the APase-APDase PhoD could be also detected in the cytoplasmic fraction. These proteins might represent the unprocessed precursors of the APases still containing the signal peptides. On the secretome map, the APase PhoB and the APase-APDase PhoD belong to the most prominent extracellular proteins induced after phosphate starvation. The major APase, PhoA, was identified only as a minor phosphate starvation-induced protein probably because this enzyme has high specific activity (Antelmann *et al.*, 2000). APase-APDase PhoD is believed to cleave the phosphodiester bonds of teichoic acids (Eder *et al.*, 1998). Besides these phosphate starvation-specific proteins, the  $\sigma^B$  regulon is induced after phosphate starvation. The  $\sigma^B$ -dependent general stress proteins are expected to provide nonspecific, multiple, and prospective stress resistance to nongrowing *B. subtilis* cells in anticipation of future stress (Hecker *et al.*, 1998). Mutation in the *phoR* gene not only abolishes the transcription of Pho

regulon genes but also causes the superinduction of  $\sigma^B$ -dependent proteins (Antelmann *et al.*, 2000).

The predicted *phoP* and *phoQ* gene products consist of 223 and 486 amino acids with estimated molecular masses of 25,534 and 55,297 Da, respectively, which correspond well with the sizes of the PhoP and PhoQ proteins identified by the maxicell method. The amino acid sequences of PhoP and PhoQ of *E. coli* were 93 and 86% identical, respectively, to those of *S. typhimurium* (Kasahara *et al.*, 1992). The promoter and the amino-terminal region of *phoA*, the structural gene for alkaline phosphatase of *Escherichia coli* K12, a sequence encoding the amino-terminal portion of mature alkaline phosphatase is found and it is preceded by a sequence encoding the signal peptide. The signal peptide consists of 21 amino acids, Met-Lys-Gln-Ser-Thr-Ile-Ala-Leu-Ala-Leu-Leu-Pro-Leu-Leu-Phe-Thr-Pro-Val-Thr-Lys-Ala. The translation initiation codon is GUG, which is preceded by the Shine-Dalgarno sequence GGAG. Upstream to these sequences, there is a typical prokaryotic promoter, TATAGTC for the Pribnow box. Around the Pribnow box, there are several dyad symmetrical sequences, which may probably be concerned with the regulation of this gene (Kikuchi *et al.* 1981)

### **Eukaryotic *pho* regulon**

The phosphate-starvation response is significantly more complex in yeast than in bacteria. Under phosphate-starvation conditions, *Saccharomyces cerevisiae* induces the production of three acid phosphatases (Pho5, Pho10, and Pho11), an alkaline phosphatase (Pho8), and a high-affinity inorganic phosphate transporter (Pho84) (Oshima *et al.*, 1996). A transcription factor, Pho4, activates expression of these genes, and is itself regulated at the post-translational level. The transcription factor Pho4 is known to be repressed by phosphorylation at five serine residues under phosphate-fed conditions, while under phosphate-starved conditions this phosphorylation disappears and Pho4 (along with an additional factor, Pho2) binds to the promoters of *pho* genes (Oshima *et al.*, 1996). This phosphorylation is done by a cyclin-

dependent protein kinase (CDK), Pho85, which is permanently complexed with the cyclin Pho80. The phosphorylating activity of Pho85/Pho80 is in turn regulated by another protein, Pho81, a CDK inhibitor whose transcription is induced under conditions of phosphate starvation (Lenburg *et al.*, 1996). The end result of this system is that when the cell experiences phosphate starvation, Pho4 is dephosphorylated and the *pho* genes are expressed. The system incorporates a positive feedback loop: transcription of the Pho81 CDK inhibitor is positively regulated by Pho4 (Oshima *et al.*, 1996). *S. cerevisiae* has promoter sequence elements in common among phosphate-regulated genes. Promoter-deletion studies by Rudolph and Hinnen (1987) showed that the yeast *pho5* promoter contains four instances of the palindromic consensus sequence AYUTGYTAATTAUCAUYUT, while the *pho11* promoter contained one copy of the sequence. Two of these elements were required for *pho5* expression: when they were removed, expression levels dropped to 0.2-0.1 times normal. These elements correspond to the sites of Pho4 binding to the *pho5* promoter.

Low Pi signal (0.2mM Pi in medium), initiates *pho81* activity which inhibits the activity of *pho80-pho85* (CDK complex), which in its active state catalyzes hypo phosphorylation of PHO4 (Schneider *et al.*, 1994) Hypo phosphorylated form of PHO4 localized to nucleus activates target gene transcription together with PHO2. The phosphate transport process in *Saccharomyces cerevisiae* is characterized by a high-affinity transport system operative at low ( $\mu\text{M}$ ) concentrations of phosphate and a low-affinity transport system operative at high concentrations (mM) of phosphate. The low-affinity system, with a  $K_m$  for phosphate of approximately 1 mM at its proposed optimum of pH 4.5, is considered to be a constitutively expressed  $\text{P}_i/\text{H}^+$  cotransporter (Nieuwenhuis *et al.*, 1984; Tamai *et al.*, 1985). In contrast, the high-affinity system ( $K_m$ , 1 to 15  $\mu\text{M}$ ) is derepressible by phosphate starvation during aerobic and anaerobic cell growth.

# MATERIALS AND METHODS

## **MATERIAL AND METHODS**

### **ISOLATION OF MICROBES**

#### **Isolation by enrichment culture technique**

*Soil enrichment:* Phosphate dissolving micro-organisms were isolated in liquid medium from the soil of Thapar Technology Campus, Patiala. Soil (1g) sample was added to a flask containing 100 ml Pikovskaya broth containing tri calcium phosphate {0.5%, w/v} as insoluble form of phosphate. After one week of incubation at 37° C, 120rpm, 5% (v/v) was inoculated to fresh Pikovskaya broth. After three such successive transfers, a loopful of the culture was streaked on Pikovskaya agar and incubated at 37 C for 3 days. On the basis of their morphology, shape of the colonies and zone of clearance, various bacterial colonies were picked up and patched on fresh Pikovskaya agar plates. The various bacterial colonies so obtained were then screened for their ability to solubilize the tricalcium phosphate in liquid medium.

#### **Screening of phosphate solubilizing micro-organisms**

The various bacterial colonies were then inoculated into 5 ml of Pikovskaya broth having 0.5% (w/v) of tri calcium phosphate in test tubes which were incubated at 37° C, 50rpm. After 24hrs 5ml of the culture in the test tube was transferred to the sterilized Pikovskaya broth containing 0.5% (w/v) tri calcium phosphate. The flasks were then incubated at 37°C at 120 rpm. At regular interval of time 5ml of culture was removed aseptically & available phosphorus, protein, pH, and phosphatase activity was measured in the sample after centrifugation at 10,000 x *g* at 25° C, by stannous chloride method

#### **Available phosphorus estimation**

After 24, 40, 48, 60hr of time 5ml of culture was removed aseptically & available phosphorus was measured in the sample after centrifugation at

10,000 x *g* at 25° C and available phosphorus was estimated by stannous chloride method. In a 9ml of water 1ml of supernatant was added, 400µl of ammonium molybdate and 50µl (1 drop from 10ml pipette) of stannous chloride were added. The samples were thoroughly mixed and absorbance was taken at 690nm against a blank without any sample, within 10-12 minutes of stannous chloride addition. The blue color indicated the presence of phosphorus in the sample. A standard curve was prepared with available phosphorus (0, 5,10,15,20,25,30 µg of P<sub>2</sub>O<sub>5</sub>) in final volume of 10ml.

### **Phosphatase Activity of Bacterial Culture**

The Pikovskaya's broth having 0.5%(w/v) TCP (pH 6.8) was inoculated as previously described. After 24, 40, 48, and 60hr of incubation 5ml of culture was removed aseptically & centrifuged at 10,000 x *g* at 25° C. Activity was measured in the supernatant, in modified universal buffer (M.U.B) at pH 7. 100µl of supernatant was added in 1.7ml of M.U.B buffer & pre incubated for 15min in shaking water bath at temperature of 37°c. Then 200µl of 0.115M p-nitro phenyl phosphates was added and mixed well. The reaction mixture was incubated at 37°C for 15min. Adding 0.5ml of 7.5N NaOH stopped the reaction. Absorbance was taken at 420nm to quantify the amount of p-nitro phenol (PNP) released {yellow color}. A calibration curve was made using 0-120µg of PNP. The enzyme activity was expressed in micromoles of p-nitro phenol released per minute per mg of protein

### **Estimation of Protein**

Supernatant (100µl) of the culture media was added in 1.9 ml of distilled water. In it added 1 ml of Biuret reagent. It was mixed well and absorbance was taken at 310nm against distilled water used as a blank. . Amount of protein was calculated by calibrating with standard curves.

## **Effect of pH on growth of microorganisms & solubilization of phosphorous**

The Pikovskaya's broth having 1%(w/v) TCP was sterilized and the pH of the broth was adjusted to different pH 7,8,9,10, and inoculated aseptically and incubated at 37°c at 120 rpm .At regular interval of time {24,40,48,60 hr} 5ml of the culture was removed from the each flask aseptically & centrifuged at 10,000 x *g* at room temperature (RT) and total protein and solubility of phosphorous were determined in supernatant as given above. For each pH separate blank was made.

## **Determination for optimum temperature & optimum pH for phosphatase activity**

### **Optimum temperature**

Optimal temperature of phosphatase enzyme was estimated by the activity of phosphatase phenol phosphate (0.115M) in the reaction volume of 2ml for 15 min. The temperature was varied between 30 to 60°C with intervals of 10°C. The activity was estimated by taking absorbance against a separate blank at each temperature.

### **Optimum pH**

The flasks containing Pikovskaya's broth of pH 7, 8, 9, 10 containing tri calcium phosphate {1%, w/v} as insoluble form of phosphate was inoculated in the same way as described previously. After regular time interval phosphatase activity was determined in the supernatant. The blank used was of same pH.

## RESULTS AND DISCUSSION

## RESULTS AND DISCUSSION

### ISOLATION OF THE PHOSPHATE SOLUBILISING MICROBES

Phosphate dissolving microbial consortium were developed in Pikovskaya liquid medium from the soil of Thapar Technology Campus, Patiala. On screening the consortium numerous colonies were found on the plates, which gave zone of clearing. Four different bacterial colonies were picked up from the plates of Pikovskaya agar showing the maximum zone of the clearing around these colonies. These four bacterial colonies were purified and named as, SK1, SK2, SK3 and SK4 respectively. These four colonies were further screened for their ability to solubilize the tricalcium phosphate and phosphatase activity.

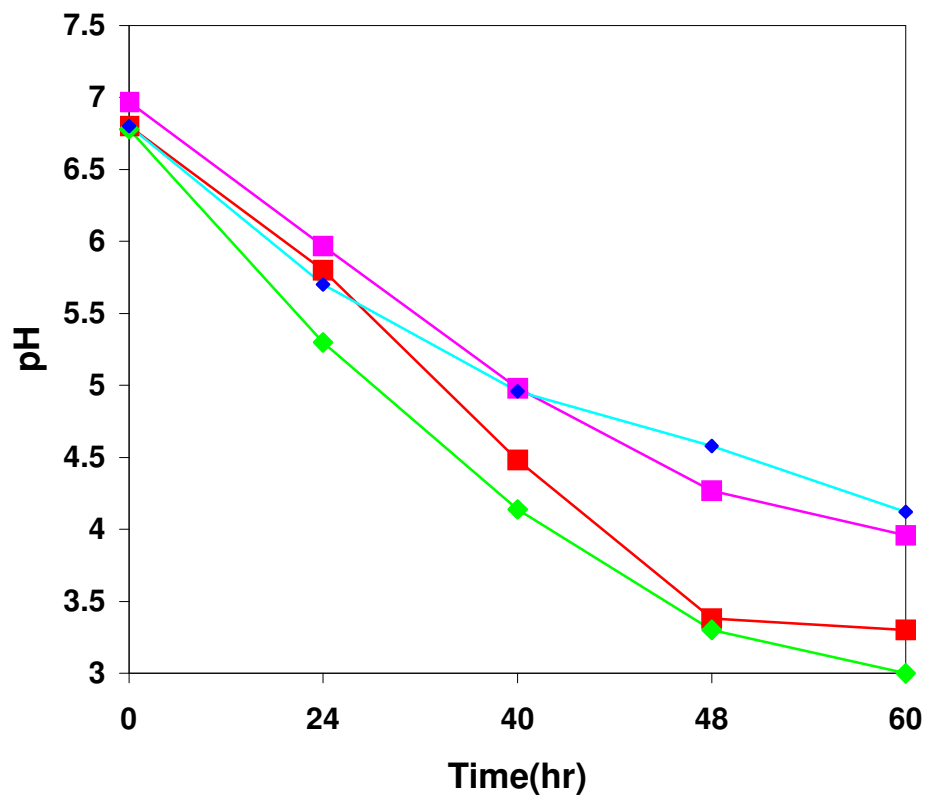
**pH of the culture media:** All the four isolates were allowed to grow on PM with TCP (0.5%, w/v). All the four culture showed decreased in pH with time. Minimum pH was observed after 60 hrs. The pH lowered down due to the liberation of the organic acids in liquid media. The minimum pH of 3 was shown by isolate SK3 after 60 hrs (Fig 1). The pH of isolates SK1, SK2, and SK4 were 3.3 ,3.96 and 4.12 respectively after 60 hrs of growth.

### SOLUBLE PHOSPHOROUS AND PROTEIN CONTENT DURING GROWTH WITH TCP

The selected isolates SK1, SK2, SK3 and SK4 were then grown on Pikovskaya liquid medium containing 0.5% TCP and their growth and pH was measured at various time intervals. All the isolates showed increase in growth up to 48 hrs. After 48 hrs there was decline in growth due low pH of the media. Soluble phosphorous content in the medium also increased along with growth. At 60 hrs soluble phosphorous content was maximum due to decrease in soluble phosphorous consumption by bacteria after 48 hrs. The isolates SK1 and SK3 solubilized nearly 30.6 and 28.5% of phosphorus respectively while SK2 and SK4 showed 24 and 26.2% of solubilization respectively (Fig 2a and 2b). The soluble phosphorus content in the medium is the difference between the solubilization of the insoluble phosphorus and

**Figure1:** pH during the growth of bacterial isolates SK1, SK2 , SK3 , SK4C in PM containing 0.5 % TCP at RT .

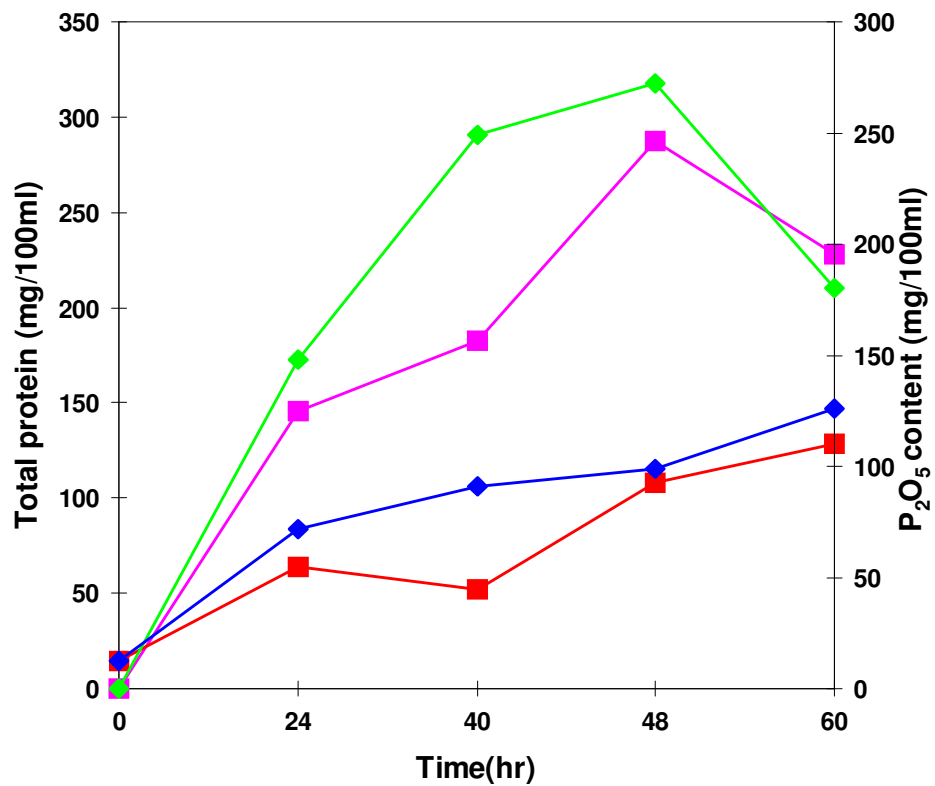
- pH SK1
- pH SK2
- pH SK3
- pH SK4



**Figure 2a:** Growth & soluble phosphorous during the growth of bacterial isolates SK1 and SK2 in PM containing 0.5 % TCP at RT with constant agitation.

Growth was measured by protein content at 310 nm. Soluble phosphorous was measured at 690 nm.

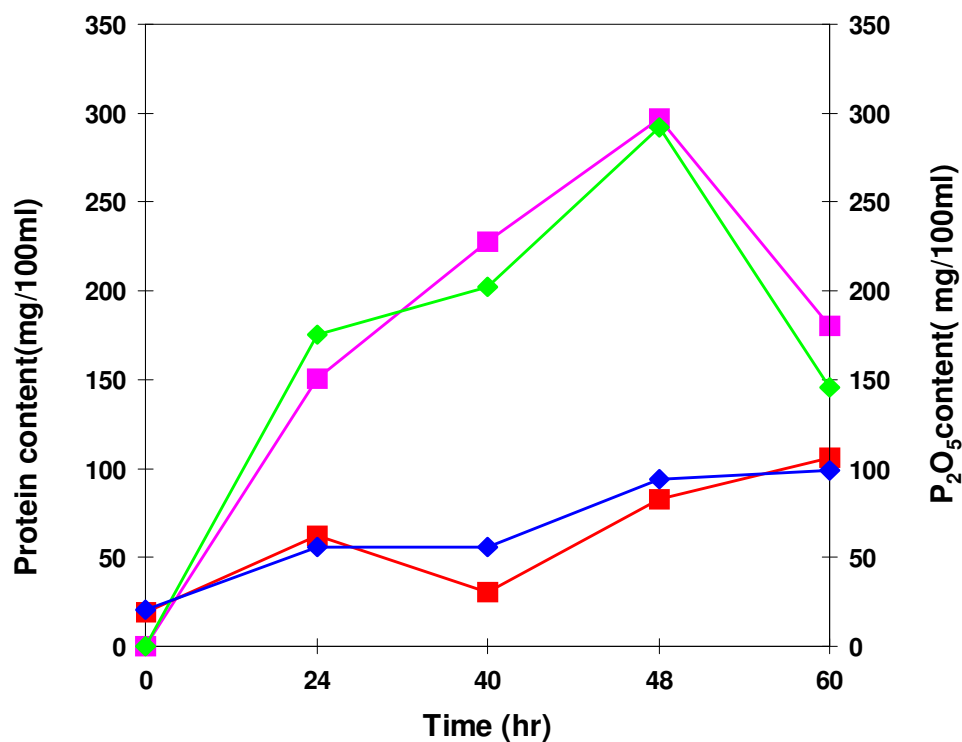
- Growth of SK1
- Soluble phosphorous of SK2
- Growth of SK2
- Soluble phosphorous of SK2



**Figure 2b:** Growth & soluble phosphorous during the growth of bacterial isolates SK3 and SK4 in PM containing 0.5 % TCP at RT with constant agitation.

Growth was measured by protein content at 310 nm. Soluble phosphorous was measured at 690 nm.

- Growth of SK3
- Soluble phosphorous of SK3
- Growth of SK4
- Soluble phosphorous of SK4



the consumption of the solubilized phosphorus by the bacteria for their growth.

The experiment was then performed with 1% TCP instead of 0.5% TCP with all the isolates at pH 7.0. Isolates SK1 and SK2 showed increase in growth with time, which showed sharp decline in growth after 48 hrs (Fig 3a). As the growth progressed soluble phosphorous content increased. After 24 hrs isolate SK2 showed maximum growth while maximum solubilization was observed by it after 60 hrs. Isolate SK1 showed decline in soluble phosphorous content after 40 hrs (Fig 3a). After 60 hrs SK1 and SK2 showed only 12 and 13 % solubilization respectively.

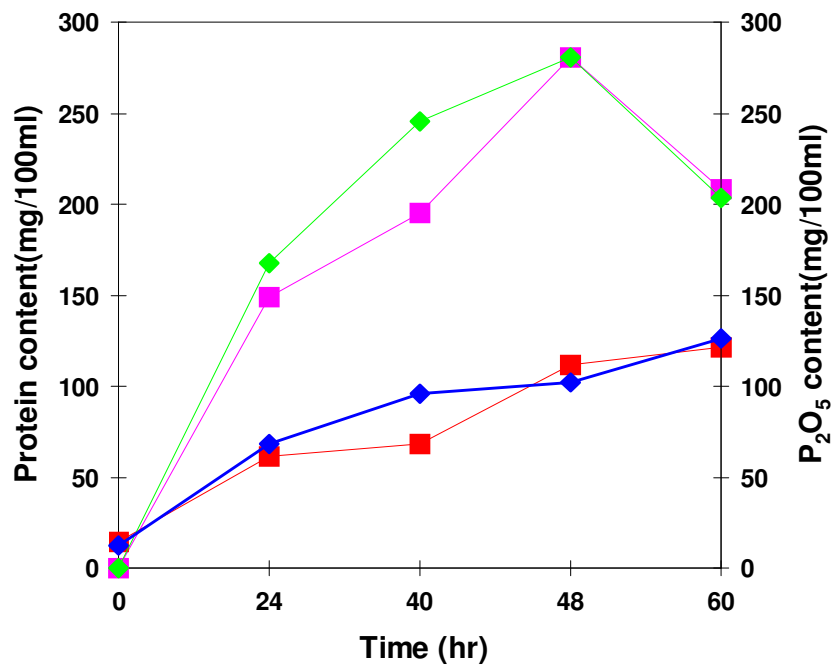
Isolate SK3 and SK4 also showed regular pattern of growth (Fig 3b). Up to 48 hrs SK3 and SK4 showed increased in growth. After 48 hrs there was sharp decline in growth as in case of SK1 and SK2. Isolate SK3 and SK4 showed increase in soluble phosphorous content along with growth. After 40 hrs SK3 showed slight decrease in soluble phosphorous content (Fig 3b). The phosphorous solubilized by SK3 and SK4 was 12% and 10.75% respectively.

The next experiment was then performed with 1% TCP with all the isolates at pH 8.0. The isolate SK1 and SK2 showed more growth at this pH as compared to pH 7.0 (Fig 4a). Growth of both the isolates showed maximum growth at 48 hrs. After 48 hrs the growth of both the isolates decreased. At this pH the phosphorous solubilized by SK1 was more as compared to that at pH 7 (154.24 mg /100ml at pH 8 as compared to 114.41 mg/100ml at pH 7) after 60 hrs (Fig 4a). The other isolate SK1 showed 116.2 mg /100ml of solubilization which was less than that at pH 7.0. At this pH SK3 and SK4 also showed increased in growth with respect to time. Both cultures showed increased in growth up to 48 hrs after that there was decline in growth. Solubilization also increased as the growth progressed. The maximum solubilization was observed after 60 hrs. The isolate SK3 showed maximum amount of solubilization i.e. 158.6 mg /100 ml (Fig 4b). Isolate SK4 showed 7% (118 mg/100 ml) solubilization.

**Figure 3a:** Growth & soluble phosphorous during the growth of bacterial isolates SK1 and SK2 in PM at pH 7 containing 1% TCP at RT with constant agitation.

Growth was measured by protein content at 310 nm. Soluble phosphorous was measured at 690 nm.

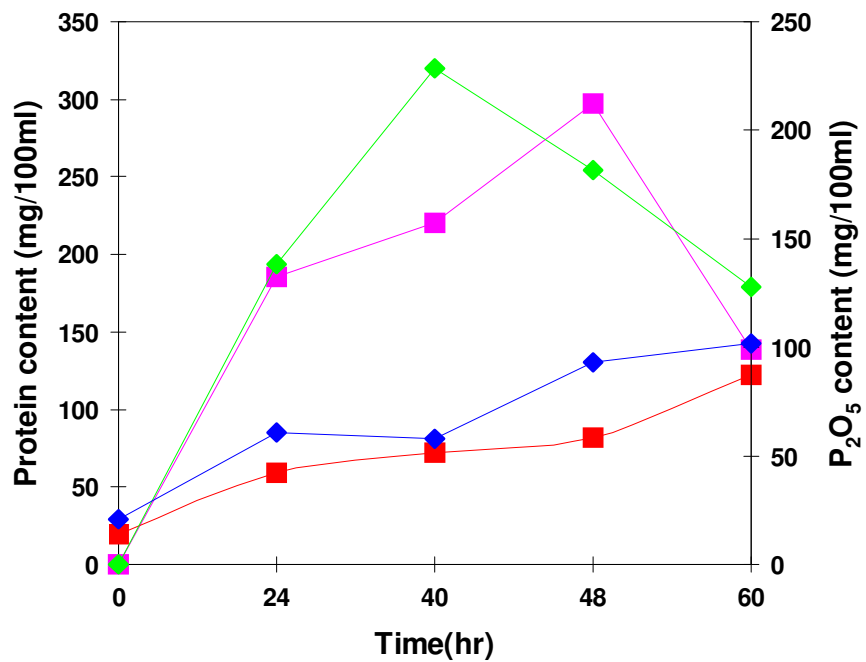
- Growth of SK1
- Soluble phosphorous of SK2
- Growth of SK2
- Soluble phosphorous of SK2



**Figure 3b:** Growth & soluble phosphorous during the growth of bacterial isolates SK3 and SK4 in PM at pH 7 containing 1 % TCP at RT with constant agitation.

Growth was measured by protein content at 310 nm. Soluble phosphorous was measured at 690 nm. Both are in (mg/100ml).

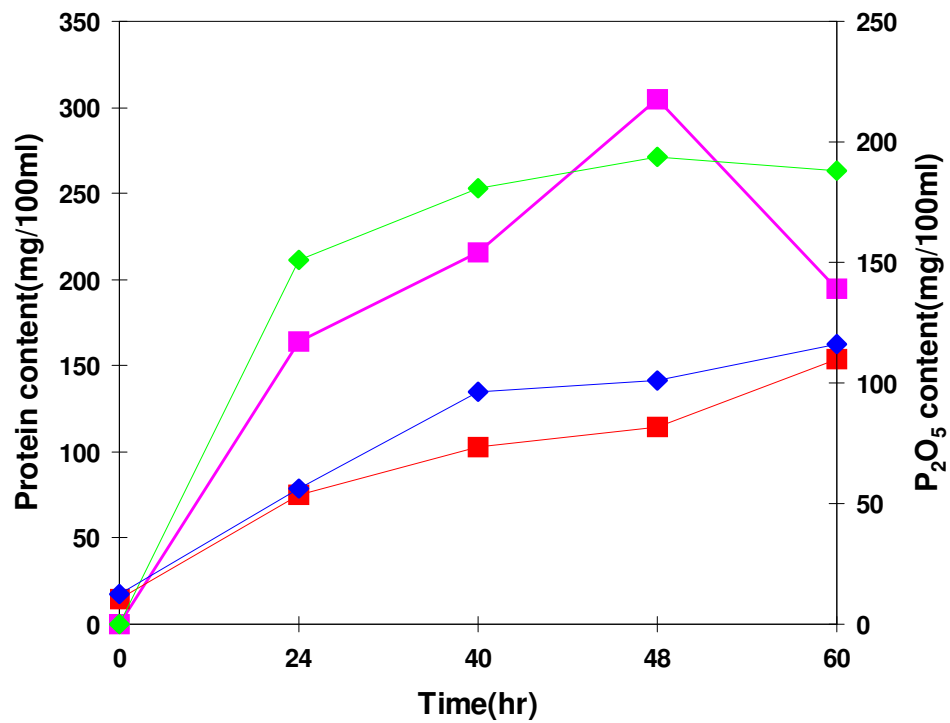
- Growth of SK3
- Soluble phosphorous of SK3
- Growth of SK4
- Soluble phosphorous of SK4



**Figure 4a:** Growth & soluble phosphorous during the growth of bacterial isolates SK1 and SK2 in PM at pH 8 containing 1% TCP at RT with constant agitation.

Growth was measured by protein content at 310 nm. Soluble phosphorous was measured at 690 nm. Both are in (mg/100ml).

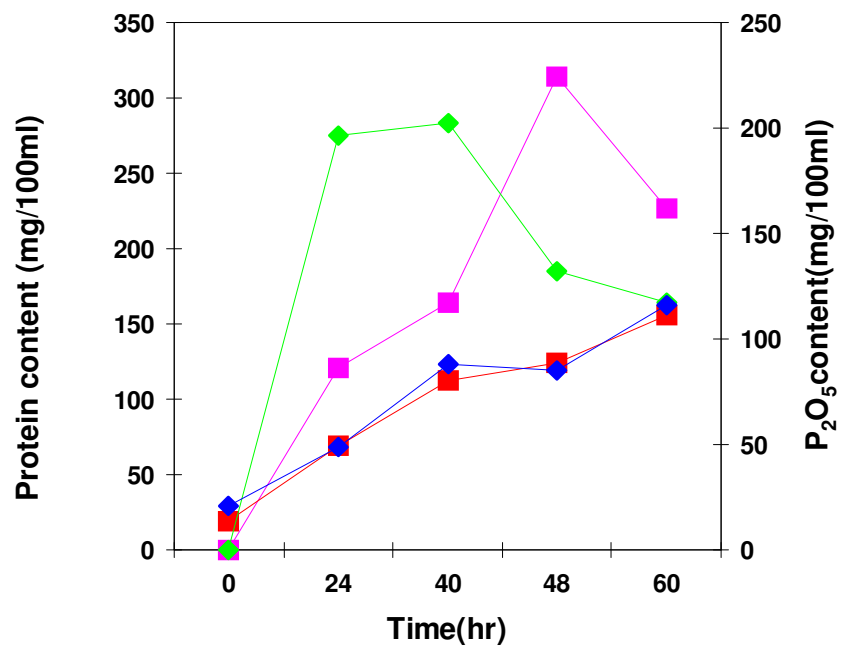
- Growth of SK1
- Soluble phosphorous of SK2
- Growth of SK2
- Soluble phosphorous of SK2



**Figure 4b:** Growth & soluble phosphorous during the growth of bacterial isolates SK3 and SK4 in PM at pH 8 containing 1 % TCP at RT with constant agitation.

Growth was measured by protein content at 310 nm. Soluble phosphorous was measured at 690 nm. Both are in (mg/100ml).

- Growth of SK3
- Soluble phosphorous of SK3
- Growth of SK4
- Soluble phosphorous of SK4



The next experiment was then conducted with 1% TCP with all the isolates at pH 9.0 to check their growth and phosphorous solubilization. At pH 9.0 only Isolates SK1 and SK2 showed growth (Fig 5) but growth was less as compared to pH 7 and 8 (Fig 4a). The isolate SK1 solubilized 96.02 mg/100ml only phosphorous (Fig 5) after 60 hrs of growth while SK2 solubilized 60.02 mg/100ml only phosphorous.

At pH10 none of the isolates were able to grow.

As we have checked the extra cellular protein secretion at different time intervals we had found that protein content was increasing up to 48 hrs and then it decreased. It means metabolic activity (protein secretion) was maximum at 48 hrs. After that the isolates entered into death phase. The pH also drops to near about 3 due to the production of organic acids or by some another mechanism. So this might be possible that the acidic range was not favourable for the growth of these isolates. This lowering of pH may inhibit the metabolic activity.

During the growth phase the microbe showed release of  $P_2O_5$  in the media. But along with this they also consumed released phosphorous for their growth and other metabolic activities. Thus the net phosphate solubilized as calculated in the medium is the difference between that consumed and that left behind. After 48 hrs growths of all the cultures declined. In this stage also they continued to release phosphorous in the media and consumption of phosphorous was less as compared to the growth phase. So after 48 level of soluble phosphorous increased while the growth decreased.

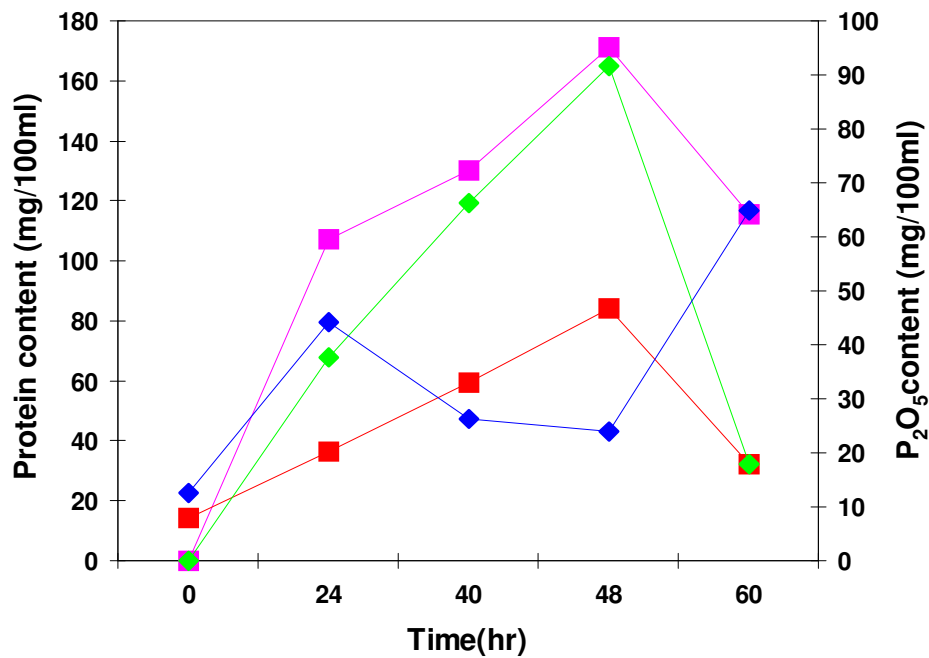
### **Mechanism of Solubilization**

Though the acid production could have taken place in our case because decrease in pH was observed in 24hrs of incubation as compared to initial pH 6.8 but after this an increase in pH was also observed after 48 hrs. As the pH of the medium did not decrease very low point but there was a slight increase in pH after 48 hrs, so the other mechanism of phosphorus solubilization could

**Figure 5:** Growth & soluble phosphorous during the growth of bacterial isolates SK1 and SK2 in PM at pH 9 containing 1% TCP at RT with constant agitation.

Growth was measured by protein content at 310 nm. Soluble phosphorous was measured at 690 nm. Both are in (mg/100ml).

- Growth of SK1
- Soluble phosphorous of SK2
- Growth of SK2
- Soluble phosphorous of SK2



be the role of enzyme phosphatase. Therefore enzyme phosphatase activity of SK1, SK2, SK3, and SK4 was estimated after growing it in PM containing TCP as sole phosphorus source.

### **Phosphatase Activity**

The selected isolates SK1, SK2, SK3 and SK4 were grown on Pikovskaya liquid medium containing 0.5% TCP and their phosphatase activity was measured at various time intervals. The phosphatase activity was estimated in the supernatant of broth taken after centrifugation at 10,000 x *g* at 4°C at various time intervals upto 60 hrs.

Isolate SK1 and SK2 showed maximum activity in the supernatant while growing in PM with TCP (0.5%)(Fig 6a). The phosphatase activity increased up to 48 hrs. After 48 hrs there was decrease in phosphatase activity. In this case maximum activity was shown by SK2 (0.0620 units) followed by SK1 SK3 and SK4. (Fig 6a). The next experiment was then conducted with 1% TCP with all the isolates at pH 7.0. At pH 7.0 isolates SK1, SK2, and SK3 SK4 showed increase in phosphatase activity with regular time interval. Isolates SK1 showed maximum activity (0.52 units) (Fig 6b) after 48 hours after which activity of the phosphatases started to decrease. This is in correlation with growth, which also decreased after 48 hours.

**Figure 6a;** Specific activity of enzyme phosphatase of bacterial isolates in supernatant in M.U.B buffer at pH 7.0 grown on (1% w/v) as sole phosphorous

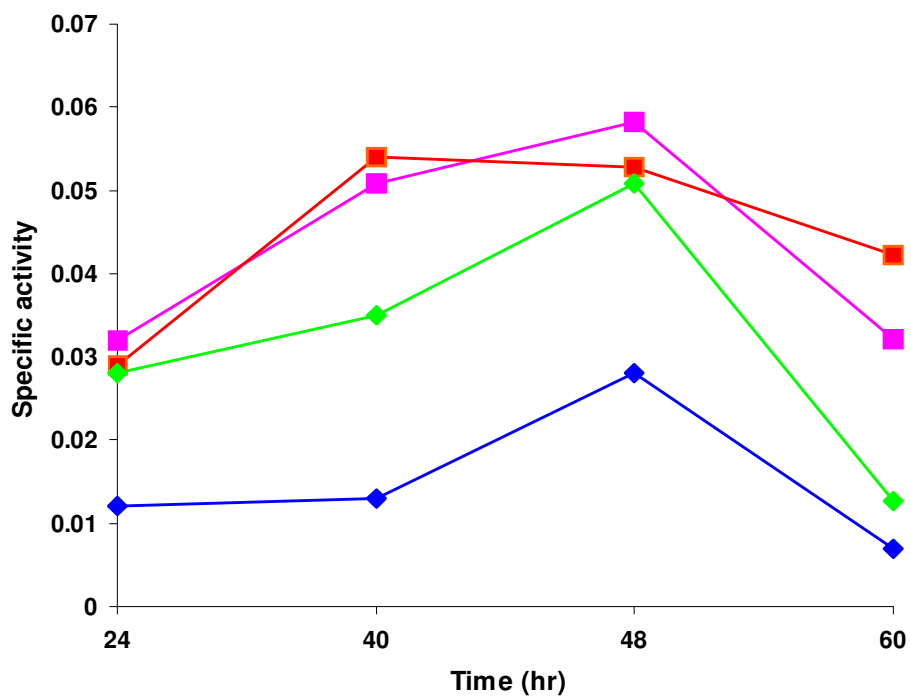
Activity was taken at 420 nm. Units of specific activity  $\mu\text{moles}/\text{min}/\text{mg}$  of protein

γ Specific activity of SK1

γ Specific activity of SK2

◆ Specific activity of SK3

◆ Specific activity of SK4



**Figure 6b;** Specific activity of enzyme phosphatase of bacterial culture in supernatant in M.U.B buffer at pH 8.0 grown on (1% w/v) as sole phosphorous

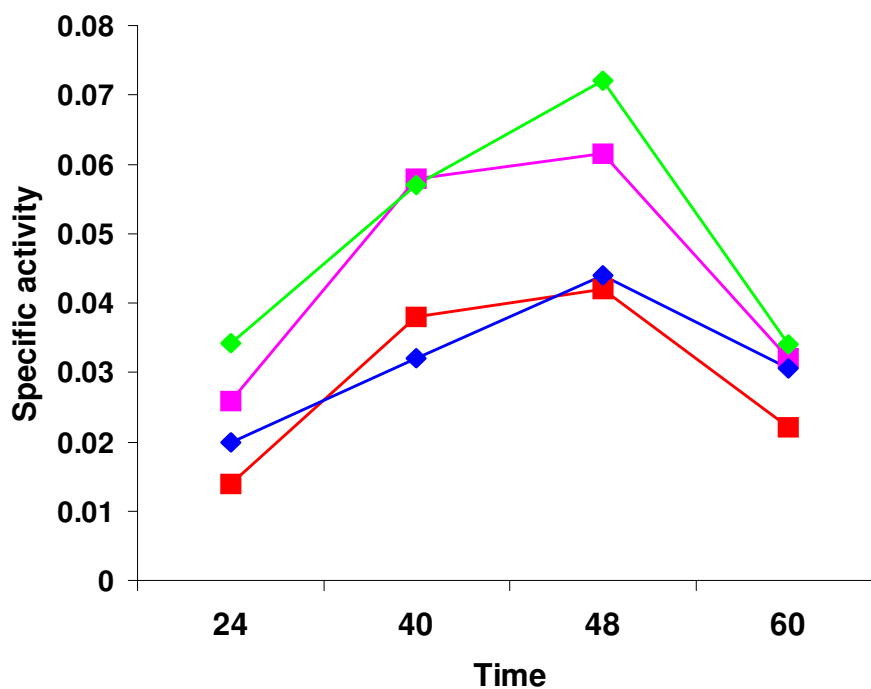
Activity was taken at 420 nm. Units of specific activity  $\mu\text{moles}/\text{min}/\text{mg}$  of protein

γ Specific activity of SK1

γ Specific activity of SK2

◆ Specific activity of SK3

◆ Specific activity of SK4



The next experiment was then performed with 1% TCP with all the isolates at pH 8.0. At pH 8 SK3 showed maximum activity (0.0712 units) followed by SK1 (0.0616 units), SK2 (0.042 units) and SK4 (0.044 units) after 48 hrs (Fig 6c). Isolate SK3 also showed maximum growth at pH 8. After 48 hrs the phosphatase activity declined.

In the next experiment at pH 9 SK3 and SK4 did not show any growth. Phosphatase activity at pH 9 of SK1 and SK2 was also very less (0.050 and 0.042 units respectively) [Fig 6d].

At pH 10 none of the isolates showed growth.

There are many mechanisms for the solubilization of phosphorous. The most possible mechanism for phosphorous solubilization was decrease in pH. The organic acids released by the isolates dissolve phosphates and the cells, which were ultimately released after the autolysis of cells, absorbed these phosphates ion. In our case growth stopped after 48 hrs and phosphorous content increased after 48 hrs. But we found best solubilization at pH 8, so the other possible mechanism was release of phosphates through the action of phosphatase enzyme in the media. The enzyme was induced only under phosphorous starvation conditions. In our all the experiments we provide external insoluble form of phosphorous (TCP), so there was no free phosphorous available for the use of bacterial growth. Under these conditions the transcription factors bind to the promoter of the phosphatase gene and induces that gene. After induction the phosphatase enzyme was liberated into the media. This enzyme acts on the dieters bonds of TCP and released phosphorous into the media. At alkaline pH our isolates SK3 and SK4 showed maximum activity.

**Figure 6c;** Specific activity of enzyme phosphatase of bacterial culture in supernatant in M.U.B buffer at pH 9.0 grown on (1% w/v) as sole phosphorous

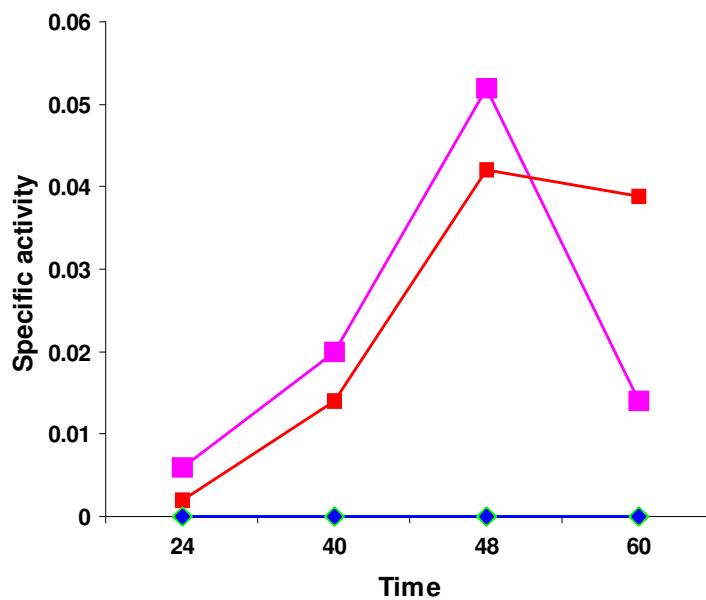
Activity was taken at 420 nm. Units of specific activity  $\mu\text{moles}/\text{min}/\text{mg}$  of protein

γ Specific activity of SK1

γ Specific activity of SK2

◆ Specific activity of SK3

◆ Specific activity of SK4



**Figure 6d**; Specific activity of enzyme phosphatase of bacterial culture in supernatant in M.U.B buffer at pH 7.0 grown on (0.5% w/v) as sole phosphorous

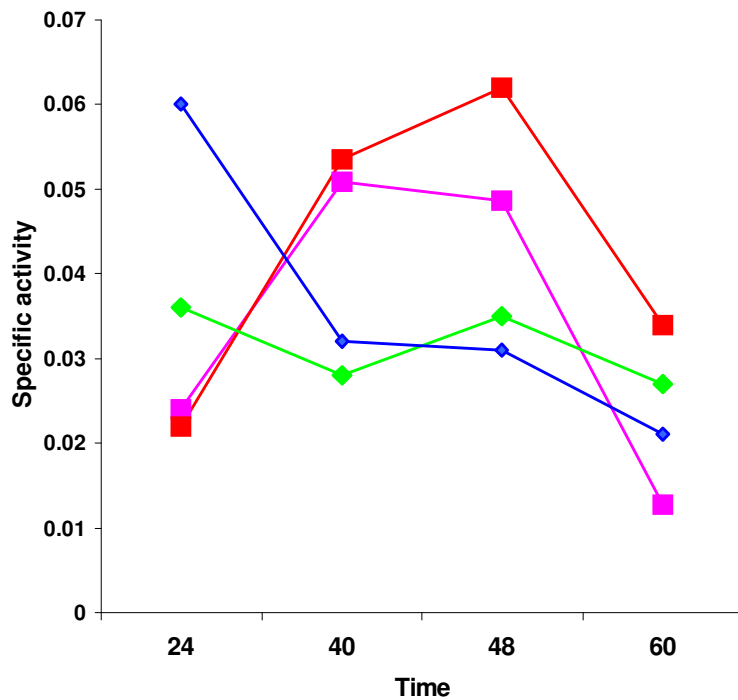
Activity was taken at 420 nm. Units of specific activity  $\mu\text{moles}/\text{min}/\text{mg}$  of protein

$\gamma$  Specific activity of SK1

$\gamma$  Specific activity of SK2

$\blacklozenge$  Specific activity of SK3

$\blacklozenge$  Specific activity of SK4



# **SUMMARY**

## SUMMARY

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- ♣ In this work 4 bacterial isolates SK1, SK2, SK3, SK4 were isolated and screened for their capacity of phosphate solubilization, growth, and phosphatase activity.
- ♣ All the 4 cultures showed maximum growth at 48 hrs and after that there was decline in growth. Isolate SK2 showed maximum growth at pH 7 while isolates SK1 and SK3 showed maximum growth at pH8.
- ♣ These isolates were able to grow in Pikovskaya medium and solubilized phosphorus as high as 30% (SK2 with 0.5% TCP at pH 7.0). Approximately 3% solubilization of phosphorus was also observed in control.
- ♣ The isolates SK1 and SK3 showed maximum solubilization at pH 8.0 with 1% TCP The soluble phosphorus content in the medium is the difference between the solubilization of the insoluble phosphorus and the consumption of the solubilized phosphorus by the bacteria for their growth
- ♣ The phosphatase activity in bacterial isolates was estimated after growing them on tri calcium phosphate as sole phosphorus source. Maximum phosphatase activity was observed in PM with 1% TCP by bacterial isolates at pH 8.0.

# **ANNEXURE**

## Annexure

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### MEDIUM USED FOR CELL CULTURING

Pikovskaya's medium, **0.5% TCP (Composition for 1 liter)**

Glucose	10gm
Yeast Extract	0.5gm
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5gm
NaCl	0.2gm
MgSO <sub>4</sub>	0.1gm
MnSO <sub>4</sub>	0.001gm
FeSO <sub>4</sub>	0.001gm
Tri Calcium Phosphate	5gm
Agar	15gm
pH	6.8

For Pikovskaya's broth agar is not added.

### CHEMICAL USED FOR AVAILABLE PHOSPHOROUS ESTIMATION

- **Ammonium Molybdate reagent:** A 25gm of ammonium molybdate was dissolved in 175 ml of distilled water. In 400ml of distilled water, 280ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added. It was mixed with ammonium molybdate and final volume was made up to one litre.
- **Stannous Chloride Reagent:** In 100ml of glycerol, 2.5gm of stannous chloride was added. It was heated in a water bath for proper mixing.

**Standard Phosphate Solution:** Anhydrous KH<sub>2</sub>PO<sub>4</sub> (96.48mg) was dissolved in final volume of one litre. It was equal to 50µg of P<sub>2</sub>O<sub>5</sub> per ml of solution.

**NaOH 7.5 N:** In 100ml final volume 30 gm of NaOH flakes were dissolved.

**NaOH 1N:** In 100ml final volume 4 gm of NaOH flakes was dissolved.

**NaOH 0.5N:** In 1000ml final volume 20 gm of NaOH flakes were dissolved. Concentrated HCl (8.8ml) was added in 100 ml of final volume.

**HCl 0.5N:** A 0.88ml of concentrated HCl was added in 100 ml of final volume.

**Stock p-nitro phenol phosphate:** A 10mg of p-nitro phenol phosphate was dissolved in 10ml of distilled water. The stock solution was kept always at 4 °c.

**p-Nitro phenyl phosphate:** The p-Nitrophenyl phosphate (4.268gm) di sodium salt was dissolved in citrate buffer, pH 4.5,25mM, to the final volume of 100ml.

#### **MODIFIED UNIVERSAL BUFFER**

Maleic acid-----2.90gm

Citric acid-----3.50gm

Boric acid-----1.57gm

NaOH 1N-----122ml

Add distilled water to make up the volume 250 ml.

**Biuret Reagent:** The  $\text{CuSO}_4$ , 0.12gm was dissolved in 20 ml of Distilled water. A 30gm of NaOH was dissolved in 50ml of water. It was cooled and mixed with  $\text{CuSO}_4$  solution in cold conditions. Final volume was made upto 100ml.

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## REFERENCES

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