ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBLIZING MICROBES

Abstract

In the present study carried out on isolation of Phosphate solubilizing capacity of microbes. The fungus isolated from soil *Aspergillus niger* and *Penecillum chrysogenum* and *Bacillus polymyxa* used for testing the effect of PH on phosphatase enzymes. The role of temperature in alkaline and acid phosphatase enzyme activity. Estimation of phosphatase enzyme activity. It is concluded the fungus *Aspergillus niger* and *Penicillium chrysogenum* shows maximum activity then bacterium.

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I. INTRODUCTION

Phosphate solubilizing microbes are increasing yield in Agriculture crops. *Pseudomonas, Micrococcus, Aspergillus, Fusarium* are the phosphate solubilizing microbes. These microbes solubilizing inorganic phosphates. So the plant absorb phosphorous easily. Phosphorous is main source of plant nutrients. These phosphate solubilizing microbes convert unavailable form of phosphorous to available.

1. **Microbial Count:** Pikovskaya's agar plates which is supplemented with phosphate. The soil samples were collected and after serial dilution technique .from the10^{2} dilution one ml of soil sample was taken and streaked on Pikovskaya's agar medium. The plates were incubated at 25^{o} C for 4 -5 days. Transparent zones of clearing around the colonies of microorganisms indicate that the phosphate present in the medium.

2. **Qualitative and quantitative measurement of phosphate solubilization in culture medium:**

   Quantitative measurement by Vanadium molydate method:

   Qualitative measurement: The pikovskaya's agar plate was prepared and well was made on the agar plate using gel puncher. Then the enzyme extracted was added onto the well at various concentration like 1µl, 3µl, 5µl, 7µl and 9µl and observed for the zone around the well.

3. **Extractionof phosphatase enzyme:** The fungi isolates like *Aspergillus niger* and *Penicillium chrysogenum* were isolated from garden soil.

   The phosphate solubilizing microbes were grown in pikovskaya's broth for 2 weeks. The fungal filtrate was filtered through whatman No. 42 filter paper, the fungal mat was then homogenized in a mortar and pestle using 0.02M tris buffer (pH 7.5), it was centrifuged at 16,000 rpm for 20 minutes and the supernatant was collected. The bacterial culture (*Bacillus Polymyxa*, and *aerobic spore former*) were grown in pikovskaya's broth for 48 hrs. Then the broth is centrifuges at 6,000 rpm for 10 minutes. Then the pellet is treated with 0.02M tris buffer (pH 7.5) and homogenized using magnetic stirrer for 20 minutes. Then the macerate was centrifuged at 16,000 rpm for 20 minutes and the supernatant was collected.

4. **Purification of enzymes:** The enzyme extracted and taken in a conical flask. It was added 20% of ammonium sulphate salt and mix well by magnetic stirrer for 30 minutes. Then centrifuged at 4,000 rpm for 10 minutes. To that add 40°C of ammonium sulphate salt and macerate it using magnetic stirrer for 30 minutes. Again centrifuge at 4,000 rpm for 10 minutes. To the supernatant add 60% of ammonium sulphate salt and macerate it using magnetic stirrer for 30 minutes. Then again centrifuge at 4,000 rpm for 10 minutes and the pellet was collected and dissolved in tris buffer and stored at 4°C and then the dialysis process was carried out.

5. Estimation of phosphatase enzyme activity:

- **Acid phosphatase assay:** 1ml of citrate buffer prepared of pH 4 for *Bacillus polymyxa*, and pH 5 for *Aspergillus niger*, *Penicillium chrysogenum* and *aerobic spore former*. . Then 1ml of enzyme extracted.it was added to the buffer, 1ml of p-nitro phenol phosphate was added to each test tube. Then the tubes were incubated at 30°C for *Aspergillus niger*, and *Penicillium chrysogenum* and 40°C for *Bacillus polymyxa* and *aerobic spore former* for 30 minutes. To this add 4ml of 0.1N of sodium hydroxide was added and the OD value was noted at 405nm.

- **Alkaline phosphatase assay:**1ml of Sodium carbonate and bicarbonate buffer was prepared of pH 8.5 for *Penicillium chrysogenum* and *aerobic spore former*, and pH 9 for *Aspergillus niger* and *Bacillus polymyxa* were taken in separate test tubes. 1m of culture filtrate obtained was added to this buffer and mixed for 5 minutes. 1ml of p-nitro phenol phosphate was added to each tube. Then the tubes were incubated at 30°C for *Penicillium chrysogenum*, *Aspergillus niger*, and *Bacillus polymyxa* and at 40°C for *aerobic spore former* for 30 minutes. To this add 4ml of 0.1N sodium hydroxide was added and then the OD value is noted at 405nm.

II. RESULT

1. Characterization of *Aspergillus niger* and *Penicillium chrysogenum*:The *Aspergillus sp* are widespread in soil.it appeared in transparent zone around the colonies When grown on pikovskaya's agar medium, In sabouraud dextrose agar, black colonies were developed. Fig 1 A).

2. Characterization of *Bacillus polymyxa* and *aerobic spore former:* The *Bacillus polymyxa* were gram positive, rod shaped, spore- forming bacteria when observed on light microscopy (fig 1 B). Aerobic spore former were gram negative, rod shaped, spore forming, motile bacteria when observed under light microscopy (Fig 1 B).

3. The effect of pH on phosphate enzyme: In the present study Table 1 shows the acid phosphatase of *Aspergillus niger*, *Penicillium chrysogenum*, and *aerobic spore former*. The maximum activity at pH 5 PH the fungus and. But acid phosphatase of *Bacillus polymyxa* shows the maximum activity of pH 4. Graph (1) alkaline phosphatase of *Penicillium chrysogenum* and *aerobic spore former* shows the maximum activity of pH 8.5. But alkaline phosphatase of *Aspergillus niger* and *Bacillus polymyxa* shows the maximum activity at pH (Fig2).

4. The effect of temperature on phosphatase enzyme: In the present study, Table 2 shows the acid phosphatase of *Aspergillus niger* and *Penicillium chrysogenum* effective on 30°C. *Aerobic spore former* and *Bacillus polymyxa* effective on temperature 40°C. Graph (2) alkaline phosphatase of *Penicillium chrysogenum*, *Aspergillus niger* and *Bacillus polymyxa* effective on at temperature at 30°C. But alkaline phosphatase of *aerobic spore former* shows the maximum activity at temperature 40°C (Fig 2).

5. The effect of various substrates on phosphatase enzyme: In the present study table 3A, 3B, in minimal medium it contains P-nitro phenol phosphate which act as a substrate,

there was no growth observed in it. The minimal medium compared with pikovaskaya's medium (Graph 3). The pikovskaya's liquid medium showed a greater value. (Fig 2).

6. **Quantitative measurement of phosphate solubilization in culture medium:** In this study, Table 4 the *Aspergillus niger* which shows highest solubilization when compared to *Penicillium chrysogenum*, aerobic spore former and *Bacillus polymyxa*. The *Penicillium chrysogenum* shows highest solubilization when compared to *Aspergillus niger*, *Aerobic spore former* and *Bacillus polymyxa* (Graph 4).

7. **Qualitative measurement on phosphate solubilization in culture medium:** The enzymes extracted from the *Aspergillus niger*, *Penicillium chrysogenum*, *Bacillus polymyxa* and *aerobic spore former* are seen. In that it shows a very good zone of clearance. Even the 1µ of enzyme extracted showed the zone of clearance (Fig 2A).

8. **Extraction of phosphatase enzyme activity:** In our study (Fig 3 & 4), the enzymes extracted successfully from phosphatase enzyme by *Aspergillus niger*, *Penicillium chrysogenum*, *aerobic spore former* and *Bacillus polymyxa*. The enzyme was purified by dialysis method which contains 60% of Ammonium sulphate salt.

9. **Determination of molecular weight by SDS PAGE:** The bands were observed and the molecular weight was found to be 60 kilo Daltons.

III. DISCUSSION

In the present study,

Table.1 shows, Effect of pH on the phosphatase enzyme *Aspergillus niger*, *Penicillium chrysogenum* and *aerobic spore former* effective at the pH range of 5 followed by *Bacillus polymyxa* at the pH range of 4. In alkaline phosphatase activity higher at range of pH 9, lower at range of pH 8 by *Bacillus polymyxa*.

Table.2 shows, Effect of temperature on the phosphatase enzyme, in acid phosphatase *Aspergillus niger* shows maximum effect at 30^{o}C. In alkaline phosphatase the *Aspergillus niger*, *Penicillium chrysogenum* effective at 30^{o}C.

Table.3 shows, Amount of phosphatase solubilized in culture medium *Aspergillus niger* phosphatase solubilized (767µg) in tricalcium phosphate and (668µg) in monopotassium phosphate.

Table.4 shows, Estimation of phosphate solubilized by acid and alkaline phosphatase *Aspergillus niger* shows maximum acid phosphatase activity and *Bacillus polymyxa* shows minimum acid phosphatase activity.

In our present investigation shows, on qualitative and Quantitative measurement of Fungal cultures showed better activity than bacterial culture. On estimating phosphatase enzyme activity, the alkaline phosphatase enzyme showed the highest activity when compared to acid phosphatase enzyme. Phosphatase enzyme extracted and purified by Ammonium sulphate precipitation method and dialysis is carried out overnight. The molecular weight of the enzyme is identified using SDS PHAGE.

TABULAR COLUMN - 1

EFFECT OF P^H ON PHOSPHATASE ENZYME

ENZYME	ACID PHOSPHATASE				ALKALINE PHOSPHATASE			
ORGANISM/P^H	3	4	5	6	8	8.5	9	10
Penicillium chrysogenum	0.05	0.08	**0.10**	0.07	0.08	**0.11**	0.07	0.06
Aspergillus niger	0.06	0.08	**0.12**	0.09	0.08	0.11	**0.13**	0.09
Aerobic spore former	0.04	0.04	**0.06**	0.05	0.04	**0.06**	0.03	0.03
Bacillus Polymyxa	0.04	**0.05**	0.03	0.02	0.03	0.04	**0.05**	0.04

TABULAR COLUMN - 2

EFFECT OF TEMPERATURE ON PHOSPHATASE ENZYME

ENZYME	ACID PHOSPHATASE				ALKALINE PHOSPHATASE			
ORGANISM/TEMP (^{0}C)	10	30	40	60	10	30	40	60
Penicillium chrysogenum	0.07	**0.13**	0.08	0.06	0.09	**0.15**	0.10	0.08
Aspergillus niger	0.12	**0.16**	0.13	0.10	0.11	**0.18**	0.15	0.12
Aerobic spore former	0.07	0.08	**0.10**	0.07	0.07	0.09	**0.11**	0.07
Bacillus Polymyxa	0.06	0.07	**0.09**	0.04	0.08	**0.10**	0.09	0.05

TABULAR COLUMN – 3 (A)

EFFECT OF VARIOUS SUBSTRATES ON PHOSPHATASE ENZYME

59ppm stock(ml)	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
O.D	0.04	0.07	0.10	0.13	0.15	0.18	0.21	0.23	0.25	0.27	0.31	0.34	0.37	0.41	0.43
Conc(µg)	59	118	177	236	295	354	413	472	531	590	649	708	767	826	885

STANDARD VALUE (1mg = 1000 ppm)

TABULAR COLUMN – 3 (B)

AMOUNT OF PHOSPHATE SOLUBILISED IN CULTURE MEDIUM

ORGANISM	TRI CALCIUM PHOSPHATE(µg)	MONOPOTASSIUM PHOSPHATE (µg)
Penicillium chrysogenum	729	612
Aspergillus niger	767	668
Aerobic spore former	531	413
Bacillus Polymyxa	431	354

TABULAR COLUMN – 4

ESTIMATION OF PHOSPHATE SOLUBILISED BY ACID AND ALKALINE PHOSPHATASE

ORGANISM	ACID PHOSPHATASE	ALKALINE PHOSPHATASE
Penicillium chrysogenum	668	649
Aspergillus niger	708	628
Aerobic spore former	531	590
Bacillus Polymyxa	472	531

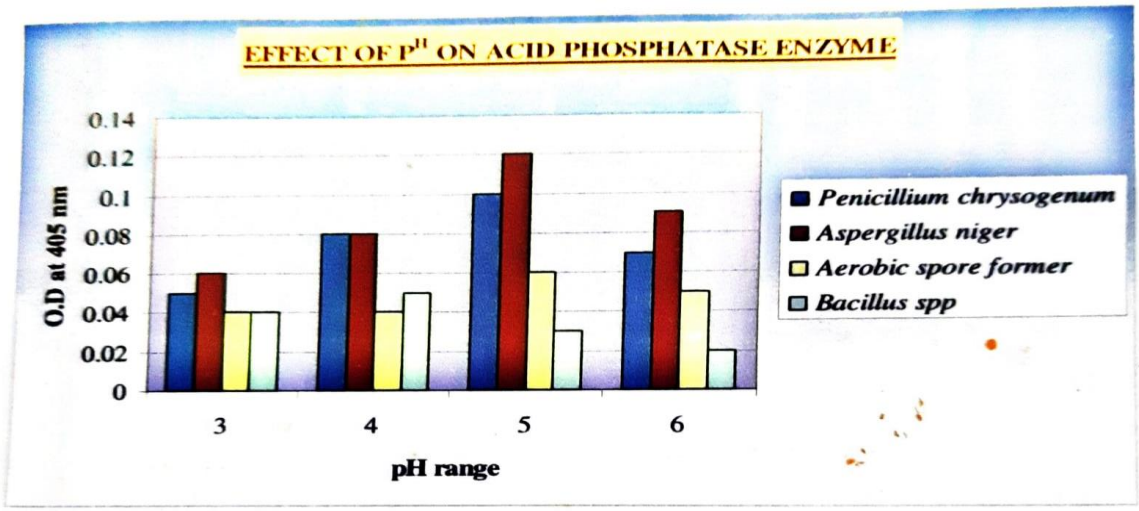
TABULAR COLUMN – 5

ESTIMATION OF PHOSPHATASE ENZYME ACTIVITY

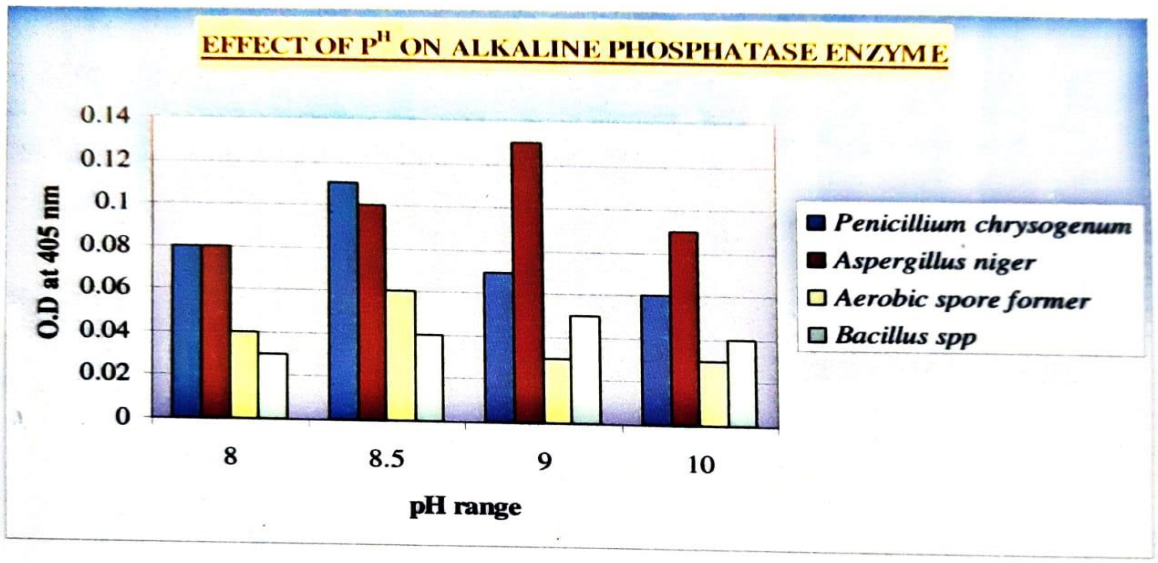
ORGANISM	ACID PHOSPHATASE	ALKALINE PHOSPHATASE
Penicillium chrysogenum	0.18	0.20
Aspergillus niger	0.16	0.21
Aerobic spore former	0.12	0.13
Bacillus Polymyxa	0.09	0.11

BAR DIAGRAM: *1*

EFFECT OF P^H ON ACID PHOSPHATASE ENZYME:

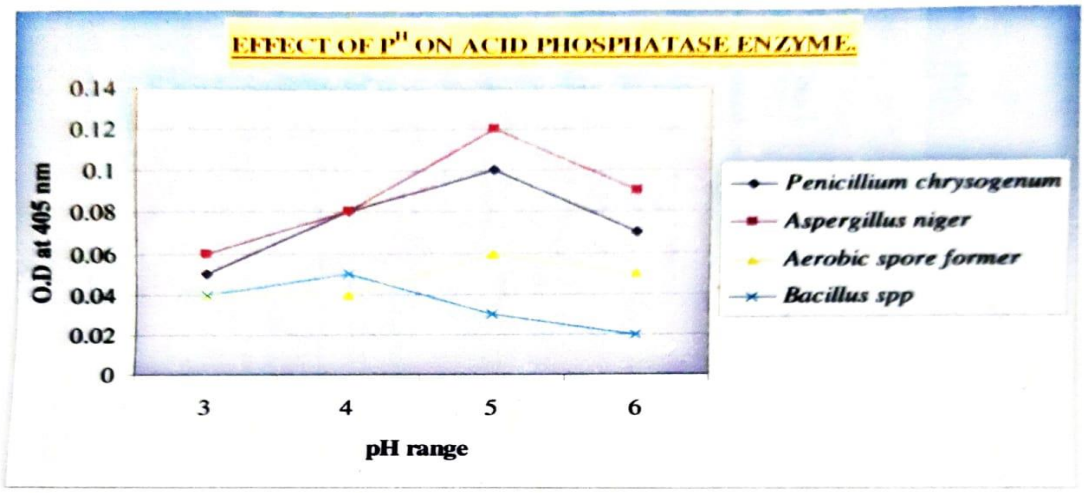


EFFECT OF P^H ON ALKALINE PHOSPHATASE ENZYME:



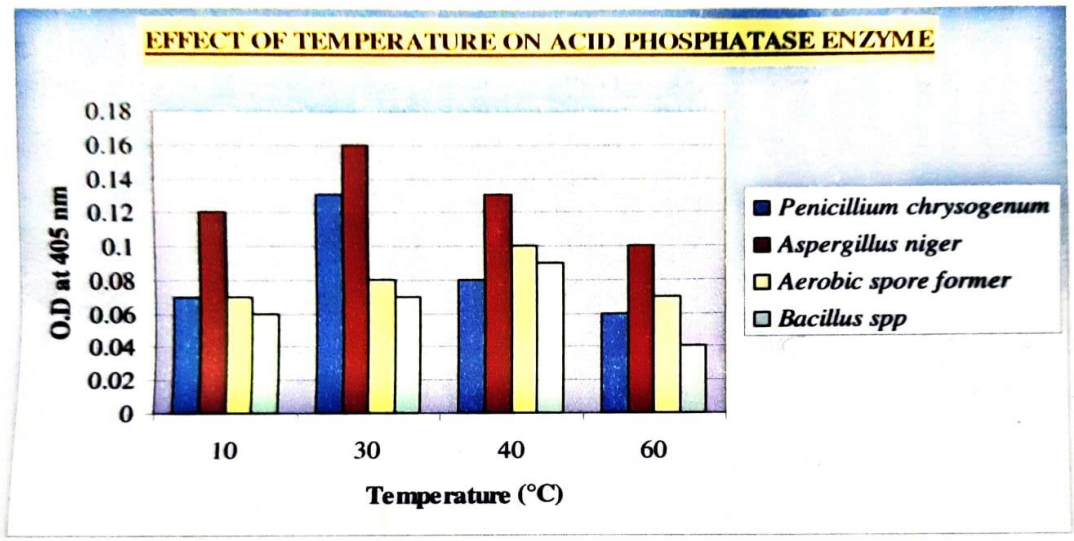
GRAPHICAL REPRESENTATION: 1

EFFECT OF P^H ON ACID PHOSPHATASE ENZYME:

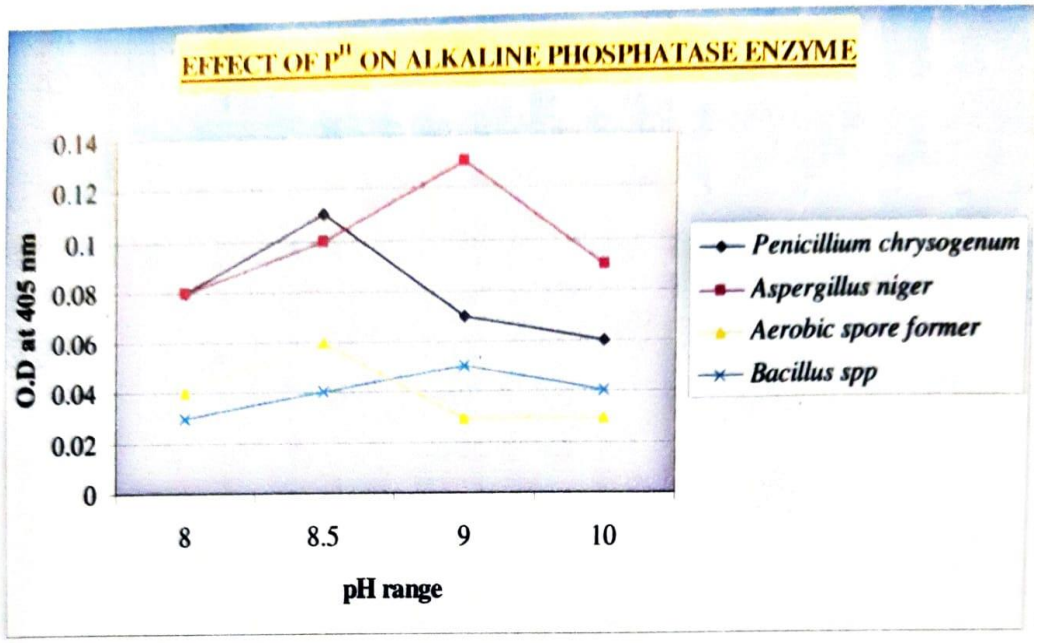


BAR DIAGRAM: 2

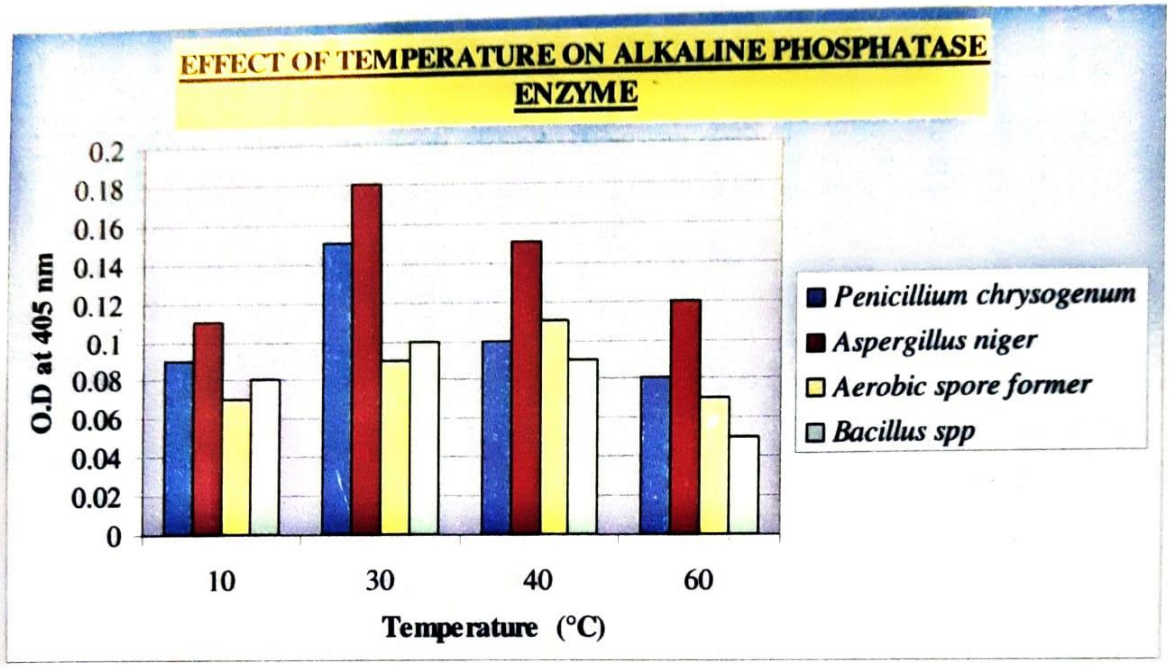
EFFECT OF TEMPERATURE ON ACID PHOSPHATASE ENZYME:



EFFECT OF P^H ON ALKALINE PHOSPHATASE ENZYME:

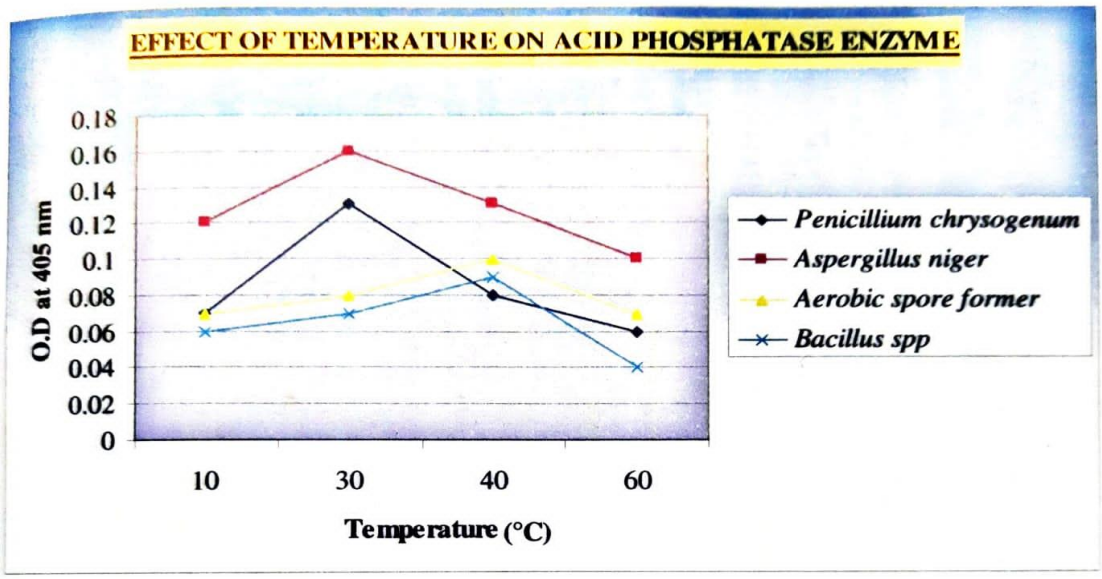


EFFECT OF TEMPERATURE ON ALKALINE PHOSPHATASE ENZYME:

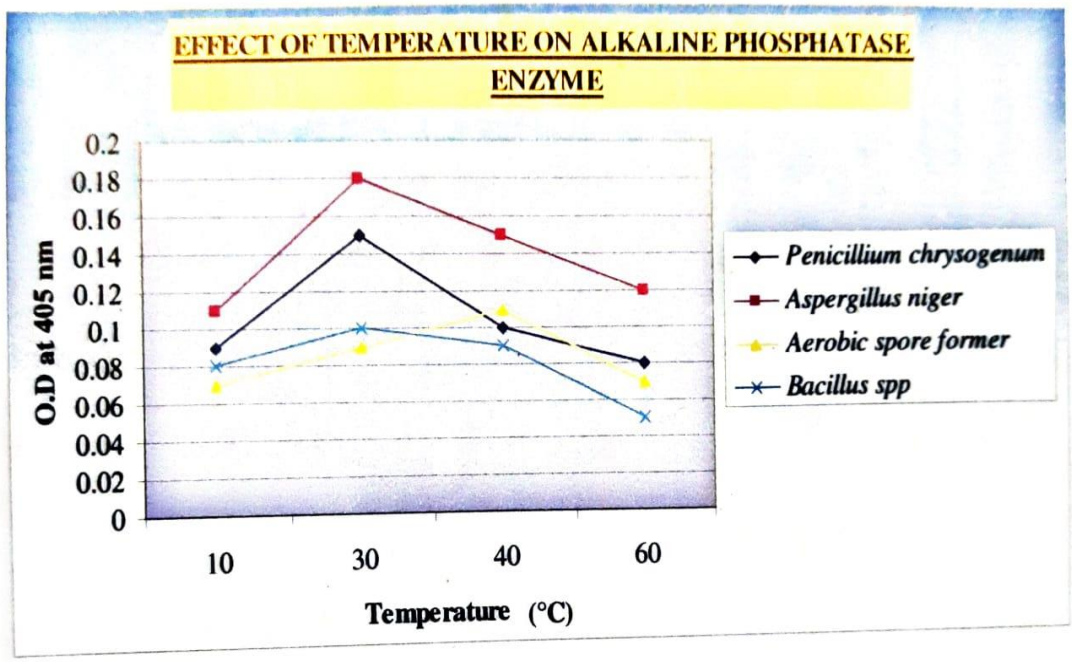


GRAPHICAL REPRESENTATION: 2

EFFECT OF TEMPERATURE ON ACID PHOSPHATASE ENZYME:



EFFECT OF TEMPERATURE ON ALKALINE PHOSPHATASE ENZYME:



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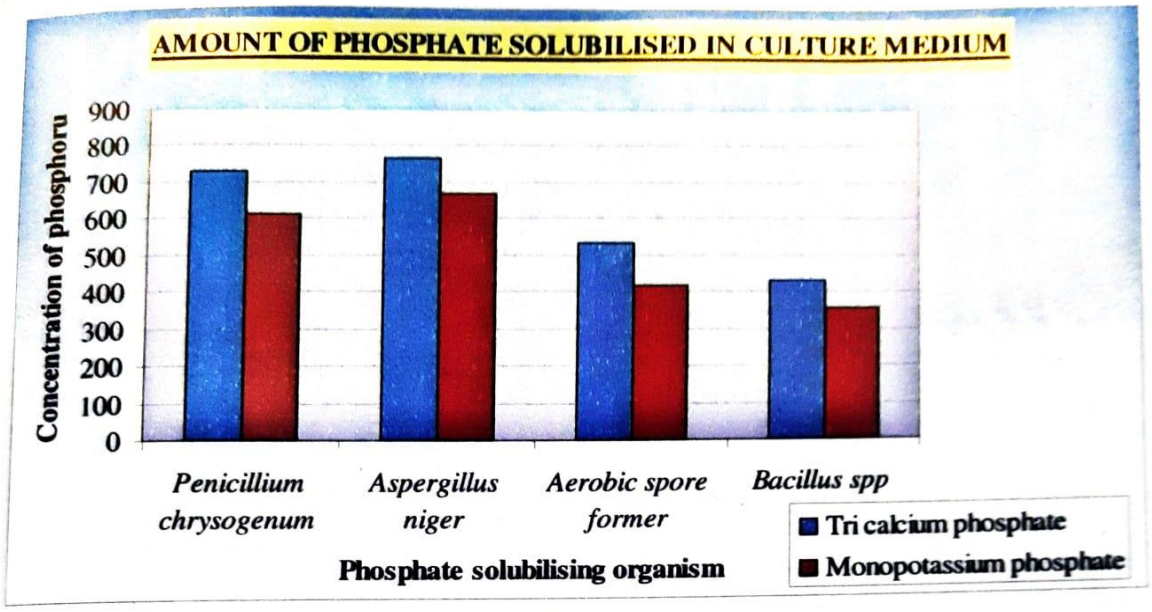

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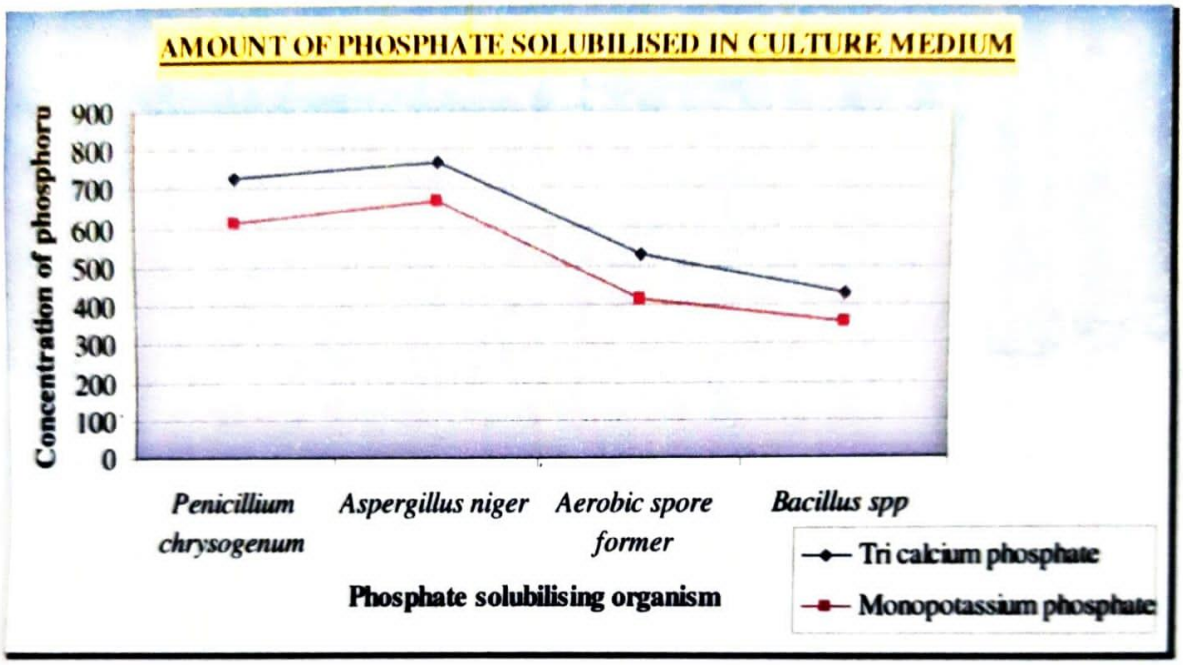

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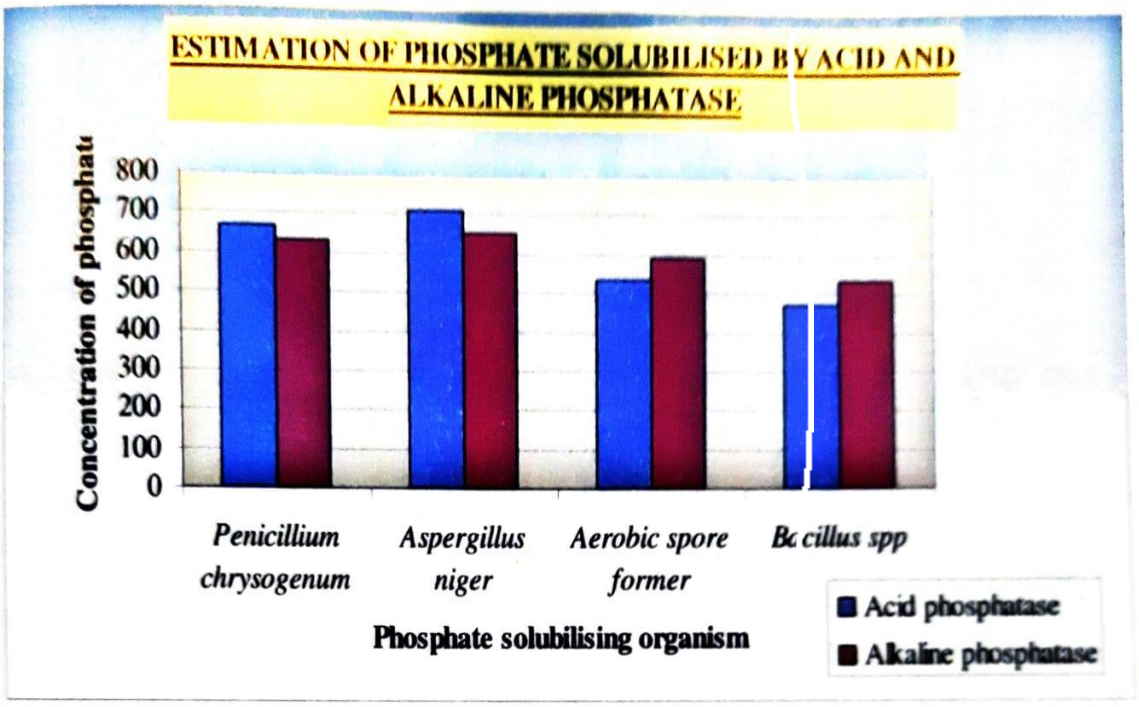

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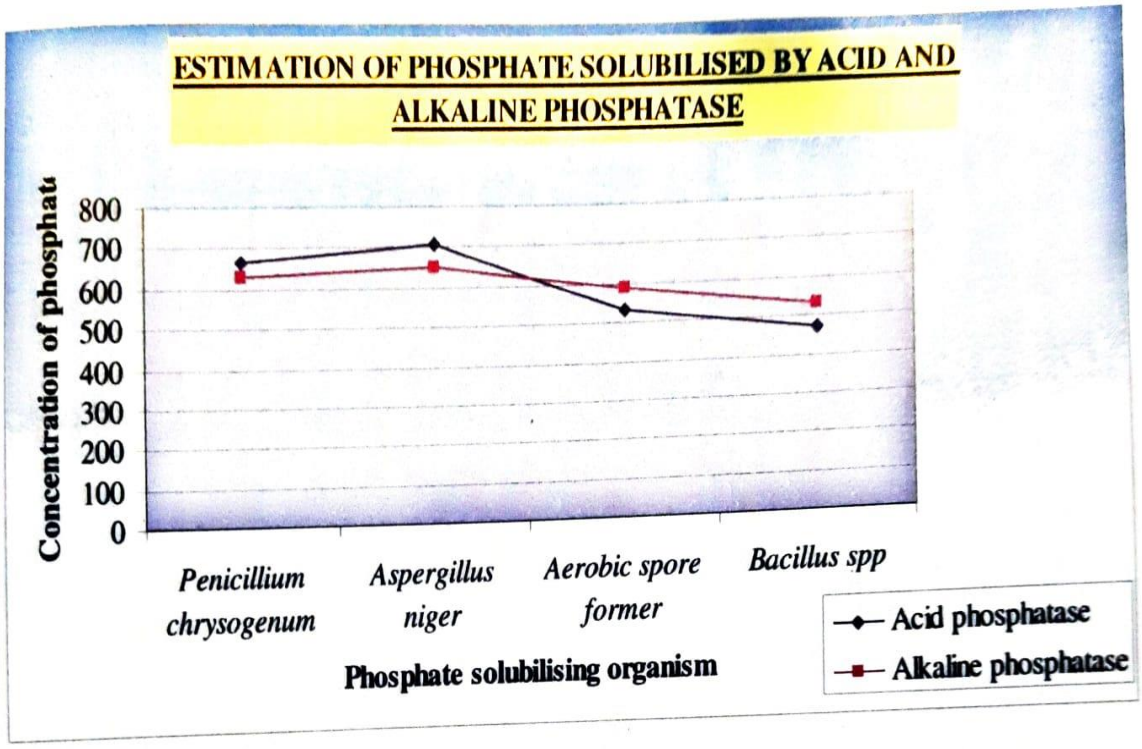

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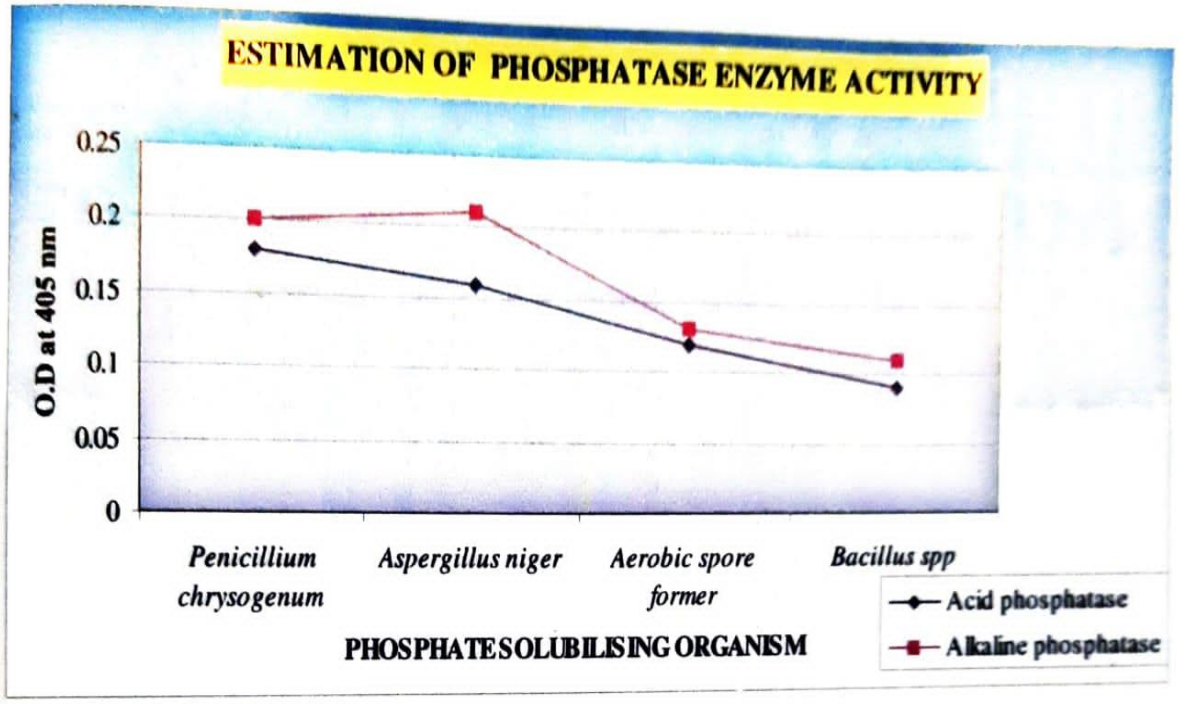

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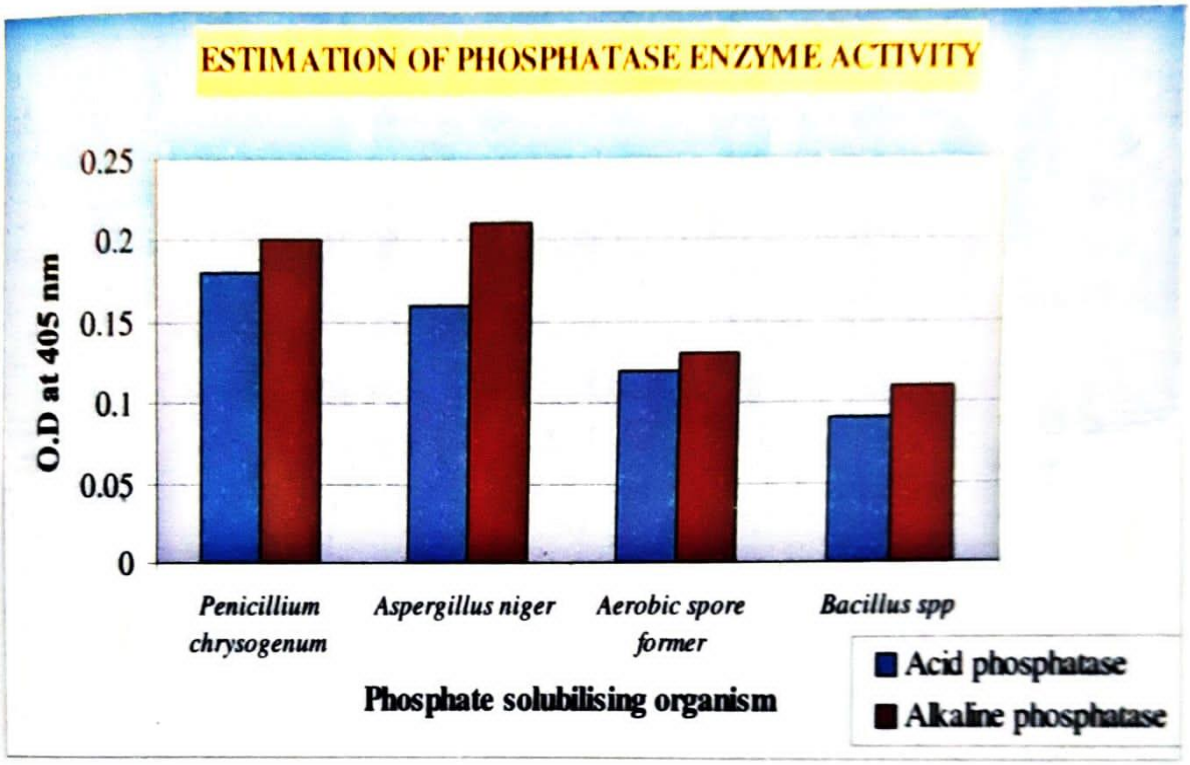
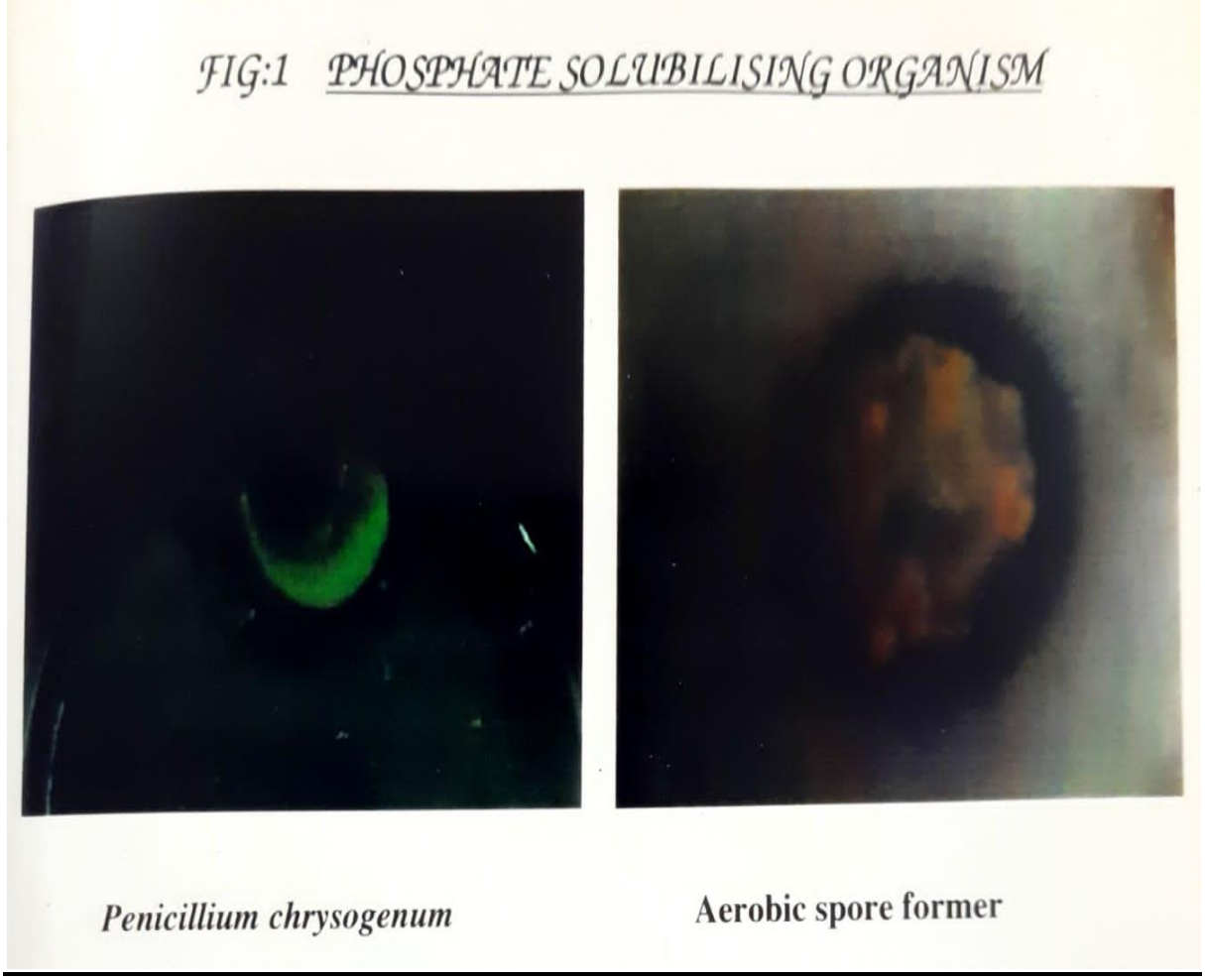
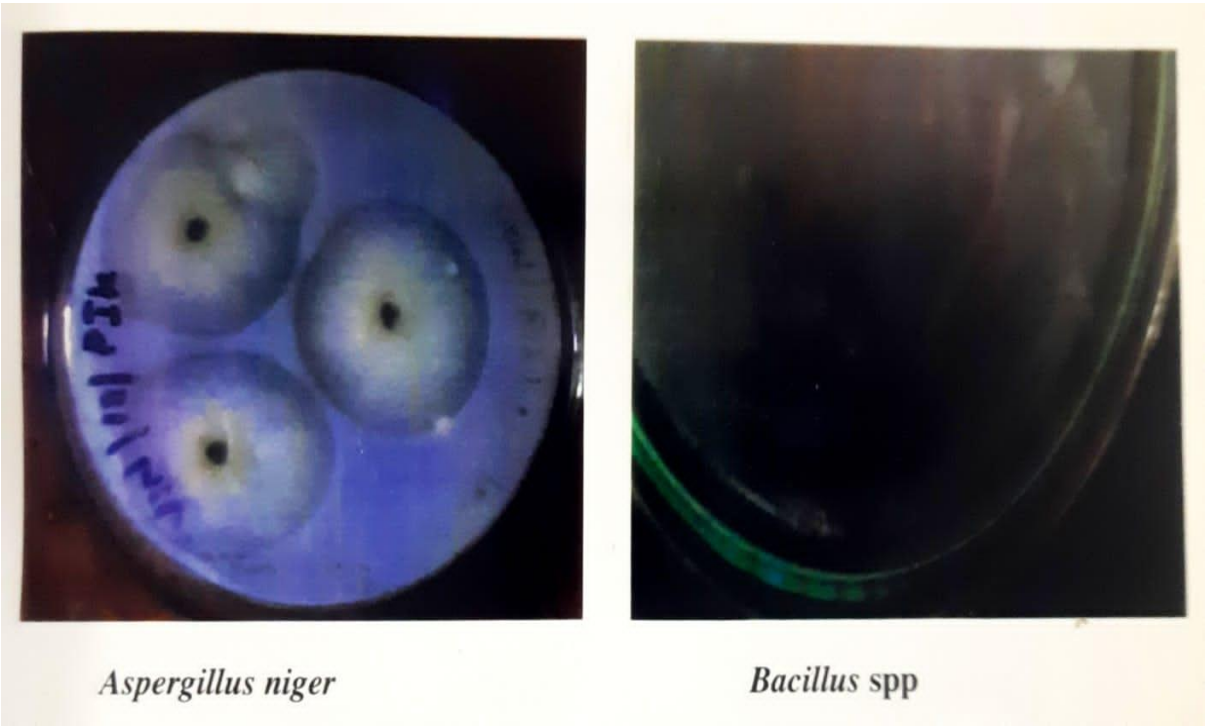


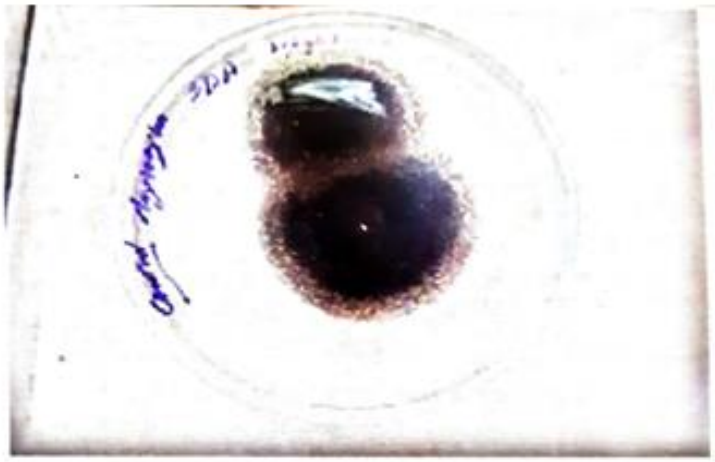
FIG:1 PHOSPHATE SOLUBILISING ORGANISM



Penicillium chrysogenum Aerobic spore former



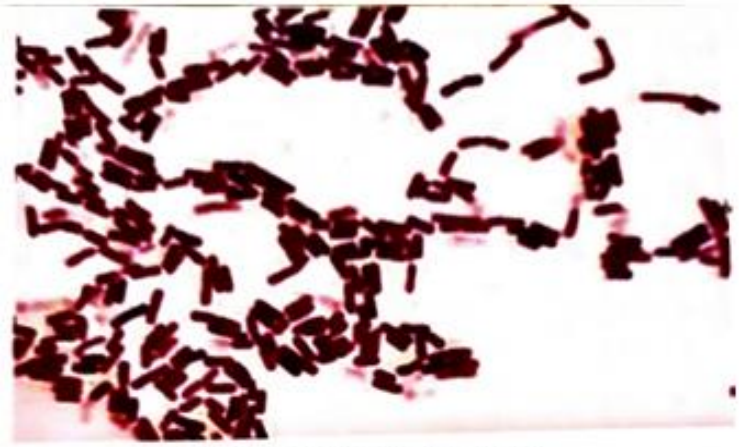
Aspergillus niger *Bacillus* spp



Aspergillus niger on SDA

P. chrysogenum on malt agar

Morphology *P.chrysogeium*



AEROBIC SPORE FORMER

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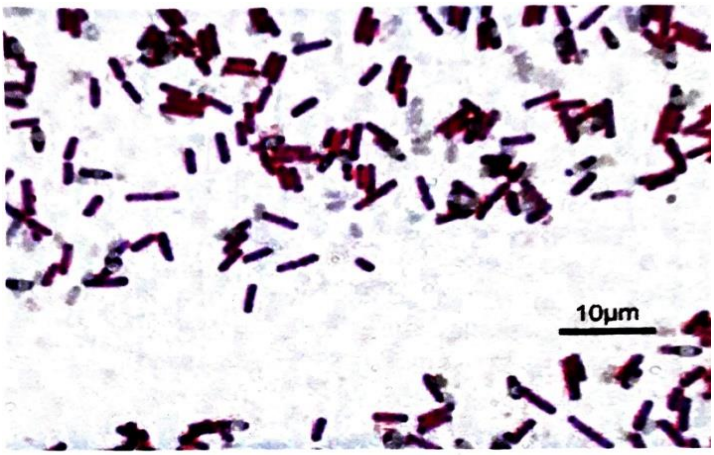
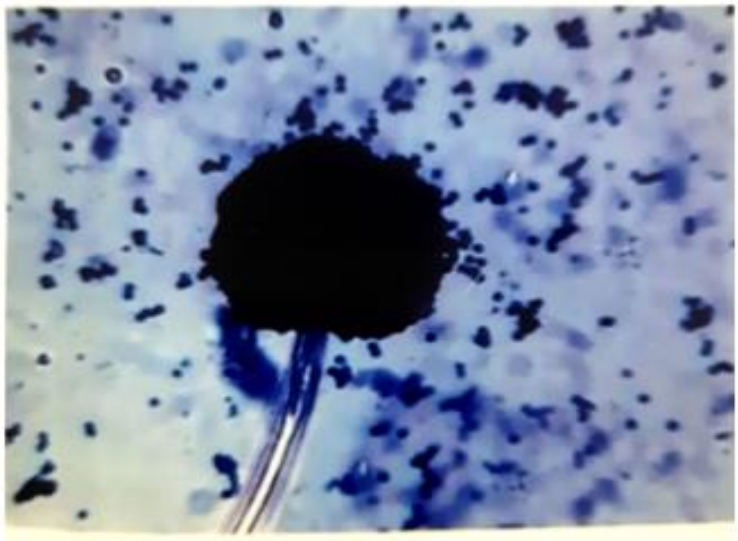
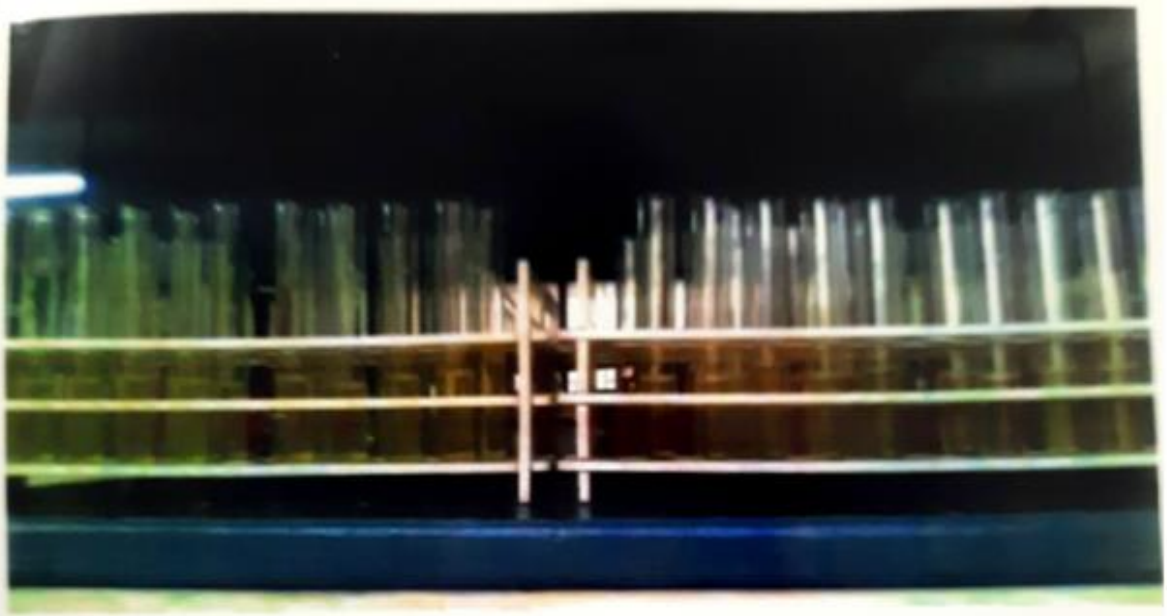


Bacillus

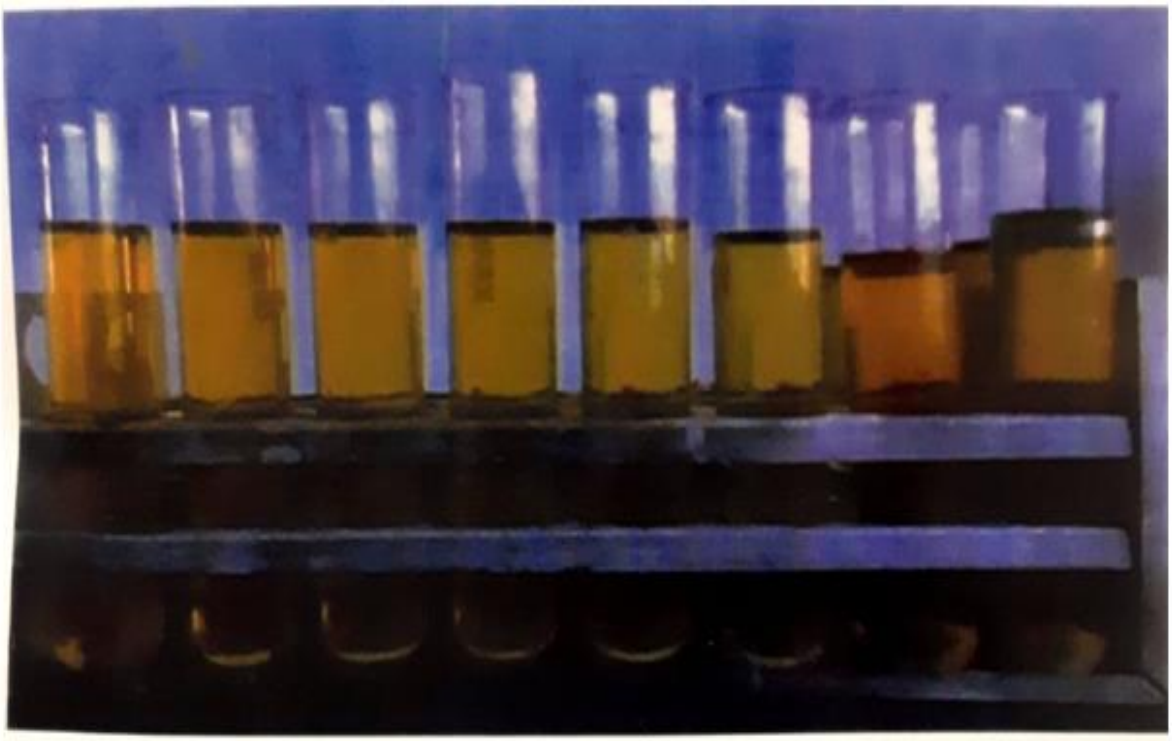


A.niger

FIG:2 PARAMETER STUDIES

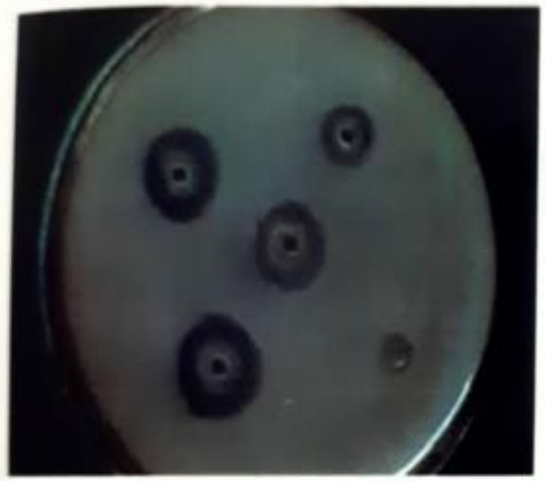


Effect of pH and temperature on phosphatase enzyme



Effect of substrate on phosphatase enzyme

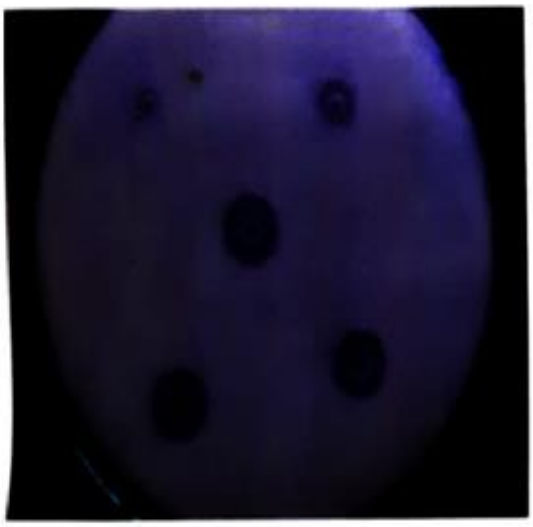
FIG: 2A QUALITATIVE MEASUREMENT OF PHOSPHATE SOLUBILISATION



Aspergillus niger

Penicillium chrysogenum

Zone of clearance around fungal enzyme extract



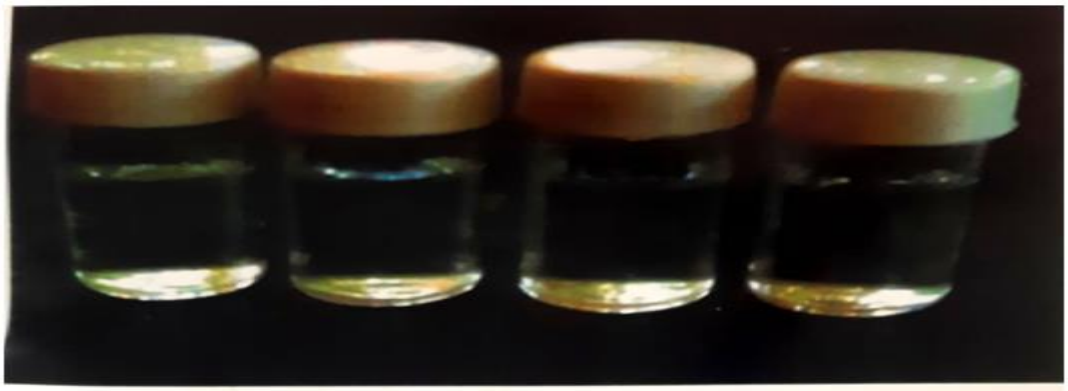
Aerobic spore former

***Bacillus* spp.**

Zone of clearance around bacterial enzyme extract

FIG:3 EXTRACTION OF PHOSPHATASE ENZYME

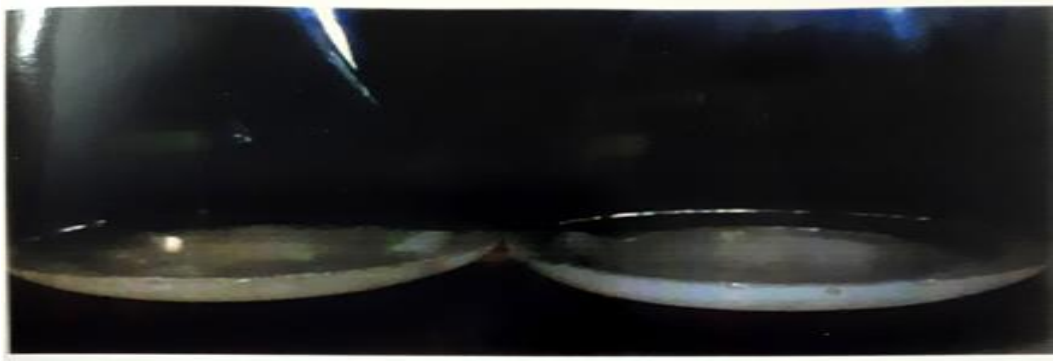
Cultures grown on pikovskaya's broth



Enzyme extract

Dialysis

FIG : 4 PURIFICATION OF ENZYMES



Ammonium sulphate purification

Determination of molecular weight by sds phage

IV. SUMMARY

Present work carried out on isolation on phosphate solubilizing microbes from soil. Microbes like *Aspergillus niger*, *Penicillium chrysogenum, aerobic spore former* and *Bacillus polymyxa* were isolated. In the present investigation the *Aspergillus niger* source highest solubilizing capacity the pikovaskaya's medium good production of phosphate solubilizing microbes. So the present work concluded that using pikovaskaya's medium for the production of phosphate solubilizing microbes.

V. REVIEW OF THE LITERATURE

[1] **Sundara Rao** and Sinha, (1963) found that the phosphate containing solid media that the microorganisms are capable of dissolving phosphates. Transparent zones of clearance, the microbial colonies indicate the extent of phosphate solubilization.

[2] **Francisco congregado et al.,** (1979) added dimethoate and marathon to the soil at 10 and 100µg/g. This caused the initial stimulation of CO_2 production. Total counts of bacterial propagates were increased.

[3] **Mukherjee and Subba Rao** (1982) proposed that the roots of higher plants provide an ecological niche to the soil microbes within the soil. This was done by genus of Pseudomonas and Bacillus.

Those bacteria are able to solubilize available forms of Fe, Ca, Mg, Al, and P. The solubilization effect is generally due to the production of organic acids. (**Kucey**, 1983)

[4] The plant growth promoting rhizobacteria (PGRR) from rhizosphere enhance the growth of plants and reduce the damage from soil bone plant pathogens (**Kloepper et al**,).

[5] The most important role of soil organism in ecosystem is decomposing of organic matters, synthesize and release them as inorganic forms that plant can use (**Setiadi et al**., 1989).

[6] **Nautical et al**., (2000) observed that PGRR are able to exert a beneficial effect upon plant growth. N_2 fixing and P- solubilizing bacteria may be important for plant nutrition by increasing N and P uptake by the plant playing a significant role as PGRR in the bio fertilization of crop.

[7] **Antananarivo Sharma et al**., (2002) done invitro studies on phosphate removal by *Citrobacter koseri* and *Micro coclus* variants revealed that they could remove phosphate upto 84 and 88% respectively from the gelatin and soap industry effluents.

[8] **Luis Henrique et al.**, (2006) isolated many enzymes produced by fungi. Isolation of filamentous fungi from the soil and humus, plant and sugarcane forty were isolated and examined for their ability to produce Xylanase, glucose- oxidase, alkaline phosphatase, acid phosphatase, phytase, pectinase, and amylase.

[9] **Stephen Joseph et al**., (2008) isolated phosphate solubilizing bacteria (PSB) possessing the ability to solubilize insoluble in organic phosphates from rhizosphere soil. The efficiency of phosphate solubilization was decreased in buffered media compared to non- buffered media.

