

**Extraction, Purification And Measurement Of Phosphate Solubilizing Activity Of Acid Phosphatase By *Aspergillus Niger* From Agriculture Soil Samples**

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**Abstract:**

Phosphatase is an enzyme that cleaves phosphoric acid monoester into phosphate ion. Phosphatases catalyze hydrolysis of its substrate so it is called hydrolase [2]. There are two types of phosphatase depend on pH alkaline phosphatase and acid phosphatase. Both enzyme are produced from fungi and bacteria. Screenings of phosphatase producing microorganisms were carried on pikovskaya agar three isolates were obtained which gave zone of phosphate solubilization on pickovaskya agar plate. By using these isolates production of enzyme was carried in pickovaskya broth. Among these three SN1, SN2, SN3, isolate SN1 gave highly production of enzyme. Protein concentration of crude enzyme was 2.13mg/ml and the purified enzyme was 0.88mg/ml. Solubilization of Phosphate was 4.82mg/ml, activity of enzyme 1.66 µg/ml/min.

**Introduction;**

Phosphorus (P) is a one of essential element for metabolic and structural function of cell, of living organism including plant and animals. [1] Generally phosphorus found in soil in form of organic form [2]. It is available in large amount in soil but it is unavailable for growing plant. As plant require inorganic phosphate for growth. The average concentration of phosphate in fertile (growing) land should be range from 5-50% [2,5].

Major four reason of Phosphorus losses from agricultural soil are. 1. Large amount of losses of soluble phosphate after use of fertilizer because of chemical precipitation or accumulation of inorganic phosphate. 2. Slowly losses of soluble phosphorus from the soil surface. 3.

Organic Phosphate released as soluble Phosphate from the rapid decomposition of plant.4. Erosion caused by wind or water.

Some free living organism (bacteria or fungi) in soil has ability to produce some extracellular enzyme like phosphatase. [8] Phosphatases are hydrolytic enzymes which catalyze cleavage of phosphoric esters (Oxygen Phosphate bond) liberating inorganic phosphate. Thus this enzyme is capable to transform or mineralization of inorganic phosphate from organic phosphate sources [3]. By this it increase the concentration of phosphate in soil for utilization of plant. Phosphatases are classified in two types, alkaline phosphatases and acidic phosphatases according to its action at particular pH [4,7]. These enzymes in soil are originated as a result of biological metabolic activity of microorganism, animal and some plants.

*Aspergillus niger* play key role in production of alkaline phosphatase in controlled environment. [7]Alkaline Phosphatase enzyme activity is affected by many factors like pH, temperature, substrate, source of nitrogen and carbon in medium or in soil [3]. Thus optimization of all this parameter is important criteria for high production and purification of acid phosphatase. [10] Enzyme production will be beneficial to not only agriculture industry but also helpful in medical diagnostic and biotech industries [6].

## **Material and Method:**

### 1. Sample collection and Enrichment of *A. niger*:

Soil sample has been collected from the fertile land of Agriculture University of Navsari, Gujarat. Sample was scrapped from 10-15 feet are from pit created at sample site in sterile polythene bags [18]. For homogenization Soil samples was suspended in D.D. water in 1:2 (wet w/v) concentration and then centrifuged at 5000 rpm for 5 min at room temperature. 1ml of Homogenized Soil sample has been inoculated in nutrient agar broth for enrichment. Sample was incubated in incubator with 120 rpm at 37°C for 120 hours [8,10,13,19]



Enrichment medium



isolation

2. Isolation of *A. niger* :

Isolation of organism was carried on Potato dextrose agar with peptone and then on pickovaskya's agar [18]. After enrichment, 1 ml from enriched broth in dilution of  $10^{-4}$  was plated on selective media like Pikovskaya's agar medium by using Pour Plate Method [12]. Poured plates were incubated for 24 to 120 hours at 30°C and 37°C aerobically. Identification of organism was carried by lacto phenol staining and confirmed by biochemical test [14].

3. Screening of phosphate solubilizing organism on pickovskaya agar plate.

Suspected colonies of isolated SN1 SN2, SN3 PSM were screened for qualitative phosphate solubilization on Pikovskaya's agar plate in duplicate. All plates were incubated at 37 °C and 30°C. The diameter of clearance zone was measured successively after 24 hours, up to 5 days. The phosphate solubilizing ratio was calculated by observing zone of clearance the observation were recorded from all duplicate plate. Species which shows clear zone around colony indicate as phosphate solubilizing microorganism [10.12].

4. Quantitative Phosphate solubilization Assay:

1ml of isolates from each isolated broth was inoculated into the Pikovskaya's broth with Tricalcium phosphate (0.3 %) in equal ratio. Inoculated broth was incubated at 37°C for up to 5 days in rotary incubator. After every 24 hours samples were collected from broth centrifuged in minispin at 10000 rpm for 5 min. Phosphorus level was analysed from supernatant calorimetrically at 410 nm using  $\text{KH}_2\text{PO}_4$  as standard. [12.14]

5. Protein estimation:

5 ml sample from each isolate of different incubation period, 24 hrs. 48, hrs. 72 hrs. 96, hrs. and 120 hrs. at 30° C and 37° C collected and filtered by Whatman no 42 filter. Supernatant used for detection of protein assay by Folin Lowry assay [13]

6. Acid phosphatase enzyme (ACP) activity assay:

ACP activity from each isolate was assayed by method of Torriani (1960). Disodium p-nitro phenyl phosphate used as substrate in this method. The amount of p-nitrophenol liberated during reaction is proportional to ACP enzyme activity which was measured in spectrophotometer at 400 nm. The entire assay was performed in three set to minimized error. [4, 12.20]

7. Optimization of pH , temperature , Incubation time and purification

Above protocol was carried out at two different temperature 30°C and 37°C, at different pH 3,4,5,6,7 and at different incubation period 24,48,72,96,120 hours for standardization to yield high level of acid phosphatase enzyme activity. Purification of acid phosphatase was done by 80% ammonium sulphate fractionation. [15,16,17].

**Result and Discussion:**

After incubation of Pikovskayas agar plate with SN1 , SN2, SN3 isolates , SN1 shows highest clearance zone which indicates that this species (isolate) produces high amount of ACP which solubilized comparative more amount of phosphate then other species.

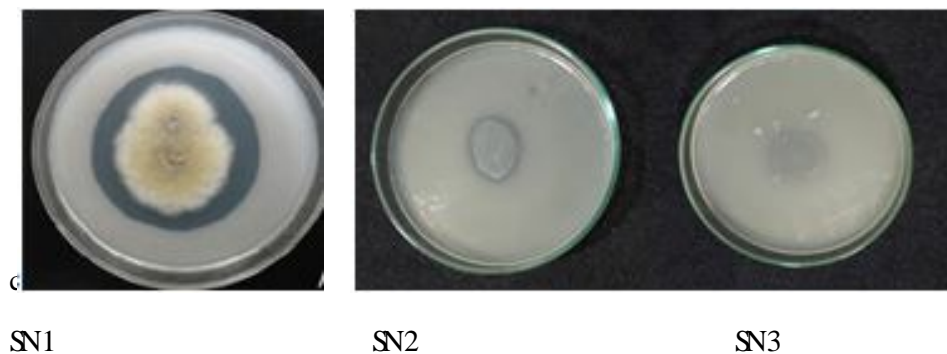


Fig. 1 zone of clearance on Pokovskays agar plate.

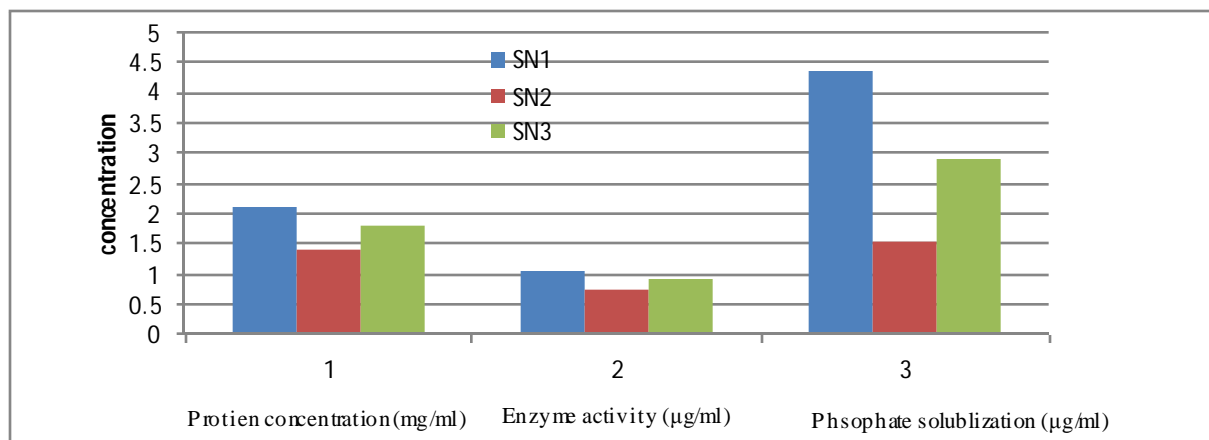


Fig. 2 Comparison of activity of SN1, SN2, SN3 for various parameter

	30°C			37°C		
	Protein concentration (mg/ml)	Enzyme activity (µg/ml)	Solubilize P concentration (µg/ml)	Protein concentration (mg/ml)	Enzyme activity (µg/ml)	Solubilize P concentration (µg/ml)
24 hour	0.51	0.77	0.66	0.93	1.10	0.72
48 hour	0.72	0.96	0.91	1.08	1.20	1.16
72 hour	1.06	1.03	1.69	1.16	1.33	3.34
96 hour	1.71	1.38	3.59	2.13	1.66	4.82
120 hour	1.32	1.15	3.18	1.06	1.27	3.66

Table 1. Optimization of Acid phosphatase at different time & temperature interval

Protein concentration, Acid phosphatase activity, solubilization of phosphate were dependent on time and temperature. Highest enzyme activity 1.66 µg/ml were observed at 37°C after 96 hours incubation. At parameter higher protein concentration 2.13mg/ml and higher phosphate solubilizing activity 4.82 (µg/ml) were observed compare to 30°C (Table 1)

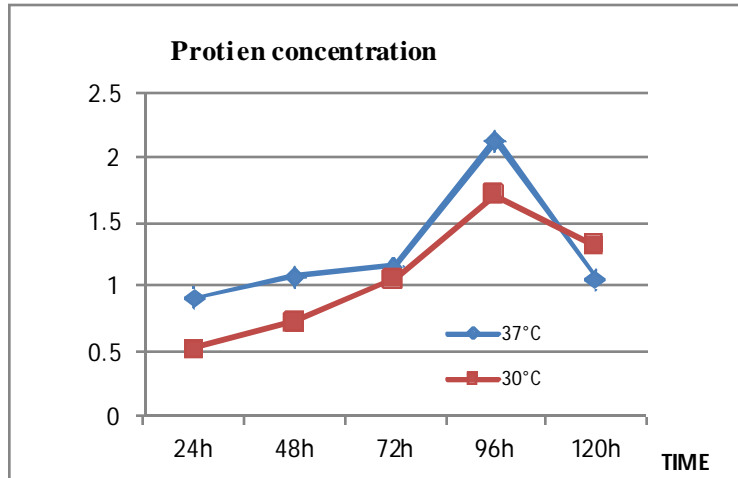


Fig. 3 Effect of temperature on protein production at different time interval

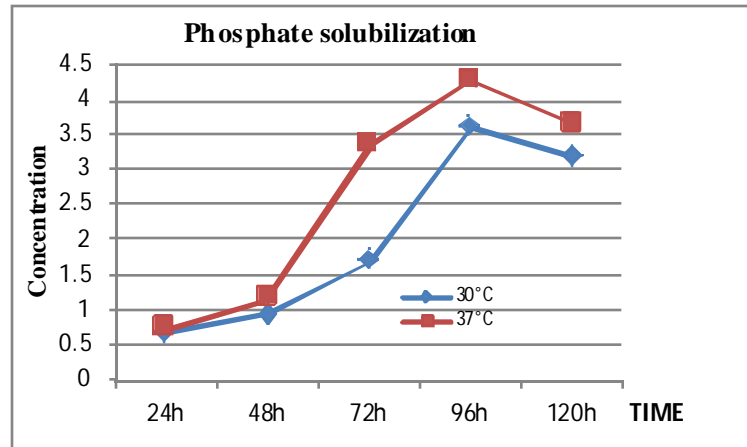


Fig. 4. Effect of temperature on phosphate solubilization at different time interval

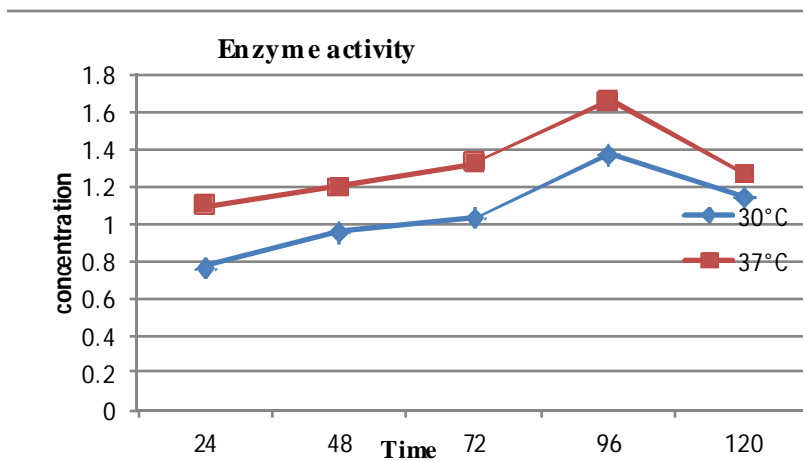


Fig. 5. Effect of temperature on enzyme activity at different time interval

pH	Protein concentration (mg/ml)	Enzyme activity (µg/ml)	Solubilize Phosphate concentration (µg/ml)
3	1.68	0.99	1.58
4	2.57	1.49	2.69
5	3.26	1.90	4.64
6	2.03	0.68	3.73
7	1.26	0.49	4.10

Table 2. Optimization of Acid phosphatase at different pH

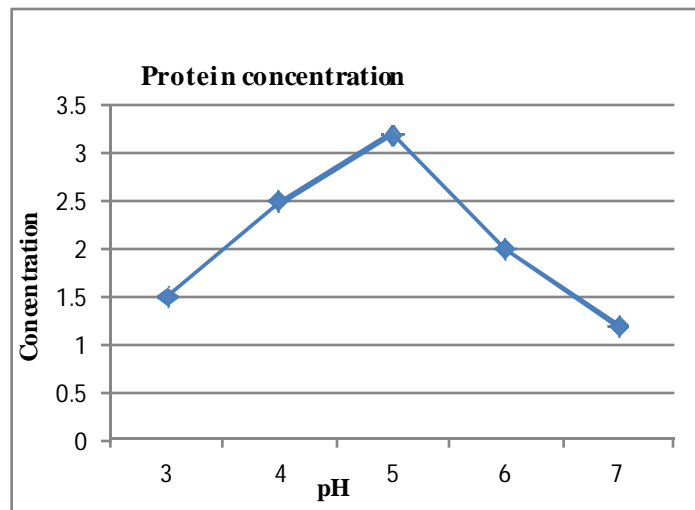


Fig. 6. Effect of pH on protein production.

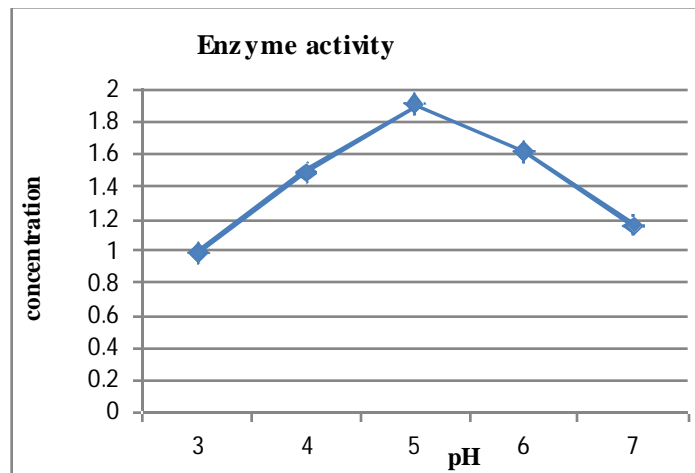


Fig. 7. Effect of pH on ACP activity.

pH play important role on activity of enzyme as well protein concentration . In study enzyme activity is increase as pH increase reached pick level at 5 pH then fall that indicates that isolated phosphate enzyme is Acid Phosphatase. Protein concentration was also increase by increased. It reached at highest level at 3.26 mg/ml at pH 5.

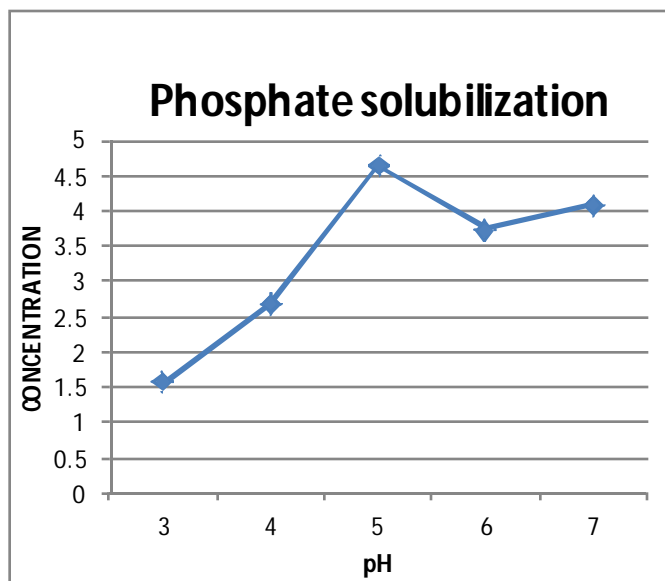


Fig. 8. Effect of pH on phosphate solubilization

Time	Protein concentration (mg/ml)	Enzyme activity (µg/ml)
24 hour	0.47	0.22
48 hour	0.70	0.41
72 hour	0.53	0.57
96 hour	0.88	0.74
120 hour	0.75	0.33

Table 3 Enzyme activity and protein secretion form partial Purified enzyme(with 80% ammonium sulphate)

After partial purification of enzyme, SN1 isolates which was incubated at 96 hours shows 0.88 mg/ml protein concentration and 0.74 µg/ml enzyme activity which were higher compare to all different time intervals. The activity of purified ACP were increasing as time increased but after 96 hours it was dropped.

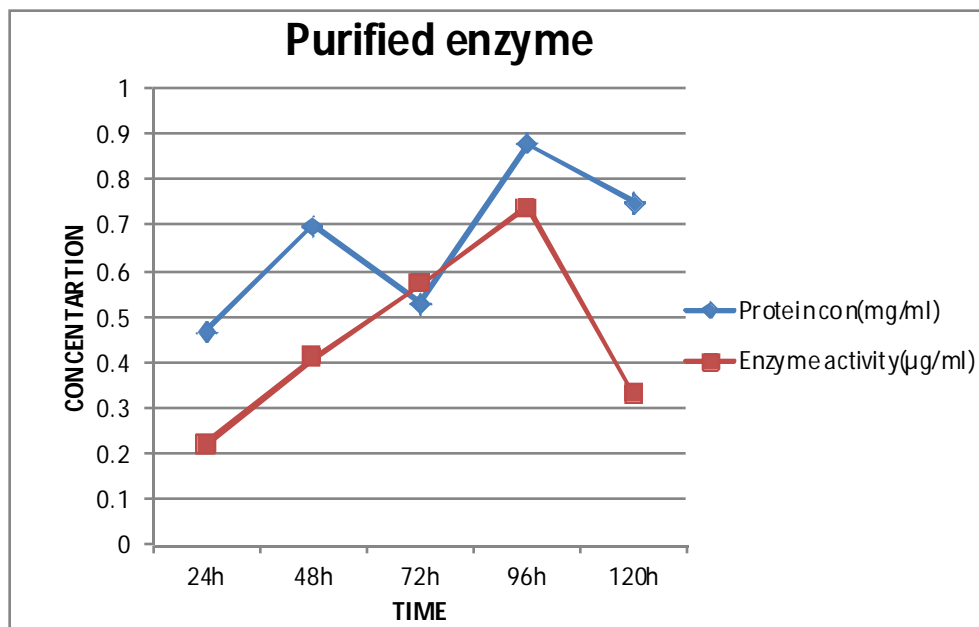


Fig. 9. Concentration of protein and purified enzyme activity in different time interval

**Conclusion:**

The phosphate solubilizing organism has capability to produce Phosphatase enzyme which able to mineralize of organic Phosphates into inorganic Phosphate. The result obtain in this study revealed that the optimum time, temperature and pH which are needed for SN1 for high production of enzyme .The SN1 highly produce Acid Phosphatase at 37°C in 96 hour at 5 pH . These results are of great interest for management of P fertilization as well in industries for commercial production of Acid Phosphatase (ACP) enzyme.

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