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Optimization and partial purification of extracellular phytase from *Pseudomonas aeruginosa* p6

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ABSTRACT

Fifty isolates capable of producing phytase were isolated from various soil samples. Out of fifty isolates five isolates showed maximum phytase activity. P6 isolate was selected for further study P6 isolate was biochemically characterized as Pseudomonas spp. The culture conditions were optimized for maximum enzyme production. The best carbon and nitrogen sources for maximum phytase production were 1% glucose and 0.5% yeast extract respectively. The enzyme was stable between the pH 4 to 10 but the optimal pH was found to be 6. The enzyme was also stable between temperature ranges 30° C to 50° C but best temperature for enzyme activity was found to be 37° C. Maximum phytase activity was 98.76 U/ml after 24 hrs of incubation under optimal conditions. The specific activity of the crude enzyme was 31.86Umg^{-1} protein and this was increased to 70.77Umg^{-1}protein by $(NH_4)_2SO_4$ precipitation.

Key words: phytase, *P. aeruginosa*, optimization.

INTRODUCTION

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental, clinical, food and pharmaceutical biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for enzymes. A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes [1].

Phytases (myo-inositol hexakisphosphate 3-phosphorylase and myo-inositol hexakisphosphate 6- phosphorylase) are classified as the family of histidine acid phosphatases, which catalyze the hydrolysis of phytic acid (myo-inositol hexakisphosphate) to inorganic phosphate and myoinositol phosphate derivatives in a stepwise manner [2]. Phytases are widespread in nature which is the main form of phosphate in foods or feeds of plant origin [3].

Monogastric animals (chickens, swine and humans) are unable to utilize phytate phosphorus either due to lack of or insufficient amount of phytate degrading enzymes. Since phosphorus is an essential nutrient for bone formation,

deficiency of this mineral would lead to osteoporosis [4,5]. The most striking chemical impact of phytic acid is its strong chelating ability with multivalent cations to form cation- phytic acid complexes [6]. The negatively charged phytic acid chelates with positively charged divalent cations rendering a poor absorption of the bound metals in small intestine [7].

Phytic acid is also partially attributed to the wide-spreading human nutritional deficiencies of calcium, iron, and zinc in developing countries where the staple foods are of plant origin [8]. Supplementation of diets with inorganic phosphorus along with the excreted phytate phosphorus, however, imposes global ecological problems when enters into rivers resulting in cyanobacterial blooms, hypoxia, and death of marine animals [9]. The supplementation of phytase in fodder improves the phosphorus bioavailability and also reduces its excretion in the areas of intensive livestock [10]. Thus, for both environmental and economic reasons phytases and phytase producing microbes are attracting significant industrial interest. Because of their great industrial significance there is an ongoing interest in isolation of new microbial isolates producing phytase and optimization of this enzyme. Hence the aim of the study is to isolate the bacteria with phytase producing ability and optimization of the enzyme production.

MATERIALS AND METHODS

Isolation of phytate degrading bacteria

Rhizospheric soil sample were collected from different agricultural crops. 1 g of soil samples was suspended in 10 ml of 0.9% saline solution and 100µl of this suspension was plated onto phytase screening medium (PSM) (1.5%glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2$ O, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 with 0.5% sodium phytate (Sigma)). The colonies exhibiting zones of clearance (translucent areas) were selected and streaked onto fresh PSM plates.

Quantitative estimation of phytate degrading bacteria

Isolates exhibiting a positive reaction were inoculated into the PSM broth with the same composition as stated above and incubated at 37 ± 1°C at 120 rpm for 5 days. Then, they were centrifuged for 10min at 10,000 rpm. The cell-free supernatant was separated and tested for phytase activity. The reaction mixture consist of 0.9 ml acetate buffer (0.2 M, pH 5.5 containing 1 mM sodium phytate) 0.1 ml of supernatant. After incubation for 30 min 37°C, the reaction was stopped by adding 1 ml of 10% tricholoroacetic acid. Assay mixture of 0.5 ml was then mixed with 4 ml of 2:1:1 v/v of acetone, 10 mM ammonium molybdate and 5 N sulfuric acid and 0.4 ml of citric acid (1M). The amount of free phosphate released was determined spectrophotometrically at 355 nm. One unit of phytase activity was defined as 1µmol of phosphate produced per min per ml of culture filtrate under the assay condition. A standard graph was plotted using potassium dihydrogen phosphate with working concentration ranging from 30 to 360 µM. Protein quantifications were made by the method of Bradford [11] and compared with the standard prepared using bovine serum albumin.

Characterization of the isolated bacteria

The bacterial isolate showing maximum phytase activity was identified upto the genus level based on the morphological and physiochemical properties of the isolate according to Bergey's Manual of Determinative Bacteriology [12].

Optimization of culture media for phytase production

Effects of various physicochemical parameters on phytase production were studied. The parameters were standardized using one factor at a time approach and by keeping the other parameters constant.

Effect of carbon and nitrogen sources on phytase production

Effect of different carbon and nitrogen sources on phytase production was determined by addition of 1% of respective sugar in place of glucose and 0.1% of different nitrogen salts to sodium phytate supplemented basal medium instead of $(NH_4)_2SO_4$. The best carbon and nitrogen sources were used in the subsequent experiments.

Effect of temperature on phytase production
To determine the optimal incubation temperature for maximum phytase production, production medium was incubated at various temperatures (25°C, 30°C, 37°C, 50°C) for 5 days keeping other conditions at their optimum level.

Effect of media pH on phytase production
To determine the effect of growth media pH on phytase production, the production media pH were varied from 5.0 to 9.0 with 1N HCl or 1N NaOH. The fermentation was carried out at 37°C keeping all other conditions at their optimum level.

Time course for enzyme production
Optimal time for phytase production at pH 6.0 and 37°C was studied by harvesting the production media at different days (0-7 days) and determining the phytase activity.

Effect of aeration on enzyme production
To study the effect of agitation speeds, flasks with production medium was incubated at static, 120 and 200 rpm.

Effect of surfactants
A level of 0.25% v/v and w/v of Tween- 80, SDS, and EDTA were separately added to phytase screen media. The medium without any surfactant served as control.

The effect of phosphate on phytase production
To study the effect of phosphate on phytase production, the activity of this enzyme in nutrient broth, medium with tricalcium phosphate and medium with sodium phytate was assayed. The three medium was incubated under same condition as above. The phytase activity was measured.

Estimation of growth
Growth was estimated by the absorbance at 600 nm. Sample from culture grown in medium with insoluble forms of phosphate were previously diluted 1:1 (v/v) using 1 N HCl to dissolve the residual insoluble phosphate and measured against a blank identically treated.

Partial purification of phytase enzyme
Partial purification of phytase enzyme was achieved by ammonium sulphate precipitation followed by dialysis. 100 ml of cell free extract was saturated with ammonium sulphate up to 60%. The content was incubated over night and centrifuged at 10,000 rpm for 10 min. Supernatant was collected and checked for enzyme activity; pellet was collected for further analysis. The enzyme mixture (pellet) was transferred to dialysis bag and immersed in Tris-HCl buffer (pH- 7) at 4°C for 24 hr.

Statistical analysis
All the experiments were carried out independently (in triplicate).The data represented here are in the form of mean ± SE.

RESULTS AND DISCUSSION

Among the fifty isolates isolated from various soil samples, five bacterial isolates showed maximum clear halo zone around the colonies on phytase specific agar medium. Out of the five isolates, isolate P6 having hydrolytic zone of 32mm (Figure 1) and showing maximum phytase activity of 15.788U/ml (Table 1) was selected for biochemical characterization. When the course of phytase production was studied in liquid medium (pH 7.2) at 37°C, the phytase activity appeared to be maximal on 3rd day of culture with a parallel decrease in pH. Culture time in the following experiments was, however, fixed to 5 days. Based on their cultural, morphological and biochemical characteristics p6 isolate was identified as *Pseudomonas* spp (Table 2).

Figure1: zones of clearance by P6 isolate on phytate screening medium (PSM).

Table 1: Qualitative and quantitative screening of different phytase producing isolates from different habitats

Isolates	Isolates	Zone of substrate hydrolysis (mm)	Enzyme activity(U/ml)mean ±SE	Habitats
P6	*Pseudomonas* spp	32	15.78±.004	Rhizospheric Soil
B5	*Bacillus* spp	17	0.19±.02	Garden Soil
P12	*Pseudomonas* spp	22	0.28±.026	Rhizospheric Soil
K9	*Klebsiella* spp	10	0.15±.001	Wheat field
P37	*Pseudomonas* spp	23	11.37±.012	Rice field

Results presented are the mean of three independent experiments with standard error values.

Table 2: Physio-biochemical characteristics of the isolate P6

Test	Observation
Gram' stain	Gram negative rods arranged singly or in pairs
Motility	Motile
catalase	+
oxidase	+
Fluorescence under UV light	+
Biochemical test	
Indole	-
Methyl red	-
Voges- proskauer	-
Citrate utilization	+
TSI	Acid slant/ Alkaline butt, gas, no H_2S
Nitrate reduction test	+
Gelatin hydrolysis	+
Urea hydrolysis	-
Starch hydrolysis	+
Sugar utilization	
Glucose	A/G
Galactose	-
Sucrose	+
Mannitol	-
lactose	-
Lysine	-
Arginine	+
Ornithine	+
Tentative identification	*Pseudomonas* spp

+ positive, - negative; A/G- acid/gas

Optimization of culture condition

It is well known that the proper optimization of process parameters plays an important role in improving enzyme yield, making enzyme production cost effective and economically feasible. Carbon is a major component of the cell

and the rate at which a carbon source is metabolized can often influence the production of metabolites. The influence of different carbon sources on phytase production was shown in Figure 2. Among different carbon sources studied, glucose showed the maximum enzyme activity of 22.165 U/ml after 48hrs of incubation. Minimum enzyme activity was observed in the presence of mannose and fructose. Easily metabolizable sugar e.g. glucose has been reported to increase phytase production by *A. niger* during submerged and/or solidstate fermentation [13, 14]. However, the effect of carbon source changes with the production strain and other conditions.

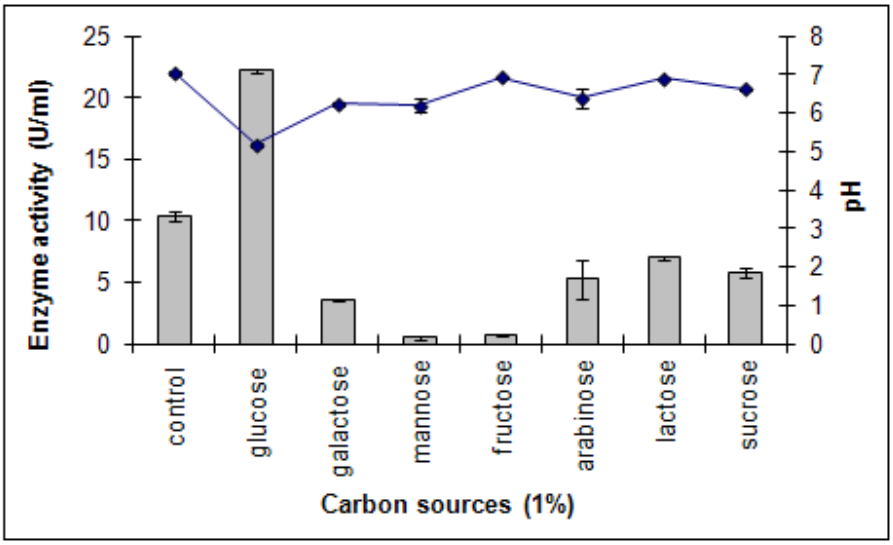

Figure 2: Effect of carbon on phytase production

To investigate the effect of nitrogen sources on the growth and production of phytase, several organic and inorganic nitrogen sources (1.0%) were added in the basal medium containing 1.0% glucose as a carbon source. Growth was similar in the media with organic and inorganic nitrogen sources (data not shown). Yeast extract showed the maximum phytase production, followed by potassium nitrate while urea did not support high phytase production (figure 3). Organic forms of nitrogen such as peptone, yeast extract have been used extensively for the production of phytase in *Aerobacter aerogenes* and *Klebsiella aerogenes* [15].

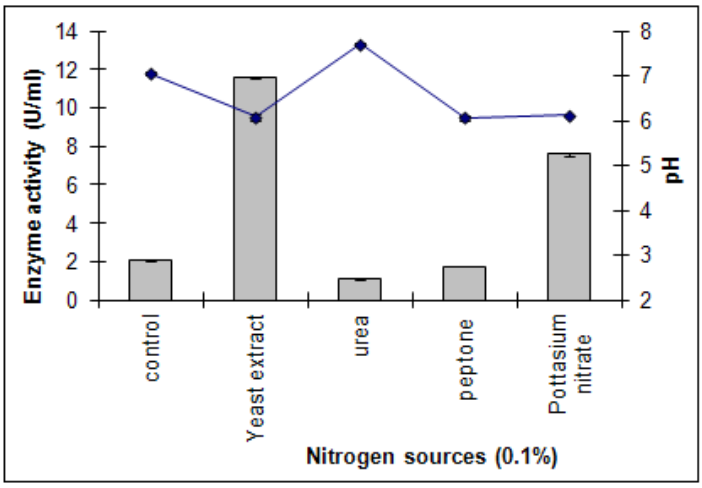

Figure 3: Effect of nitrogen on phytase production

Temperature is one of the most critical parameters to be controlled in any bioprocess. The effect of temperature on phytase production revealed that maximum yield was obtained at 37°C (29.884 U/ml) (Figure 4). The optimum temperature for the production of phytases for most of the microorganisms lies in the range of 25 to 37°C [16]. A decrease in enzyme yield was observed with further increases in temperature; hence, production of phytase by *Pseudomonas* spp was determined to be growth-related, which is a common phenomenon in many fermentation processes.

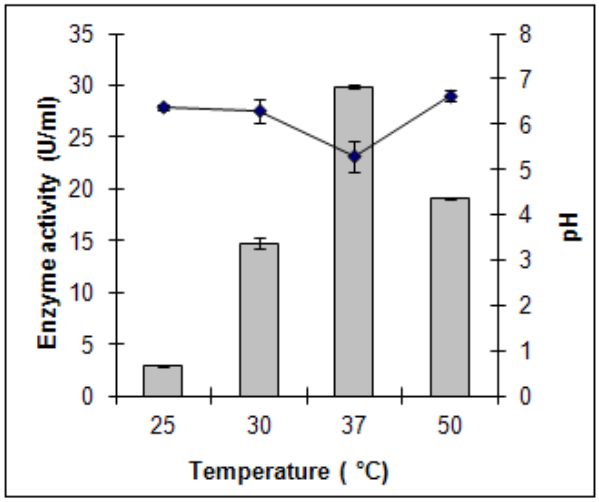

Figure 4: Effect of temperature on phytase activity

Maximum phytase production (50 U/ml) was obtained at pH 6 (30.473 U/ml) and at least 80% of the maximal activity was observed at pH values between 4.0 and 7.5. As the pH increase decrease in enzyme activity was observed and only 30% activity was noted at pH 10 (Figure 5). Increase in pH effect the charges on the amino acids within the active site such that the enzyme is not to be able to form enzyme-substrate complex. Thus, there is decrease in enzyme activity. Figure 6 show that the phytase activity increased with aeration which indicates that the organism is aerobic.

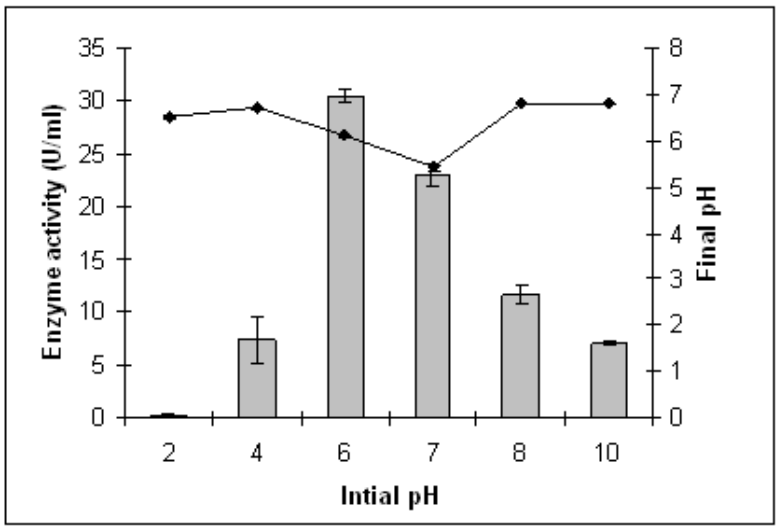

Figure 5: Phytase activity at different pH range

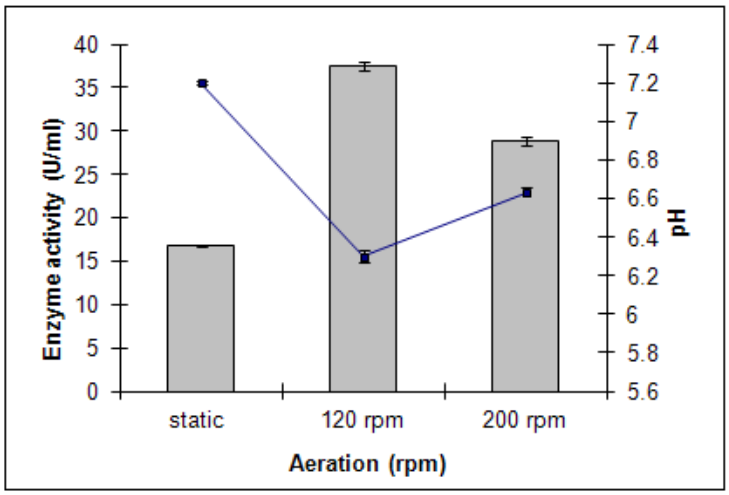

Figure 6: Effect of aeration on phytase production

When the course of phytase production was studied in liquid medium (pH 6) at 37°C, the phytase activity appeared to be maximal (80 U/ml) after 24 hours of incubation with a parallel decrease in pH. Liquid medium supplemented with SDS increased the phytase activity while EDTA & Tween-80 supported the growth but not phytase activity. Several researchers have shown the incorporation of surfactants induces the formation of smaller pellets in the liquid medium. Increase in the pelletization increases the extra cellular enzyme synthesis and hence higher yield of phytase [17-19]. Further Surfactants increases the cell wall/cell membrane permeability, which may be the reason for increase rate of metabolite secretion by the cells in the production media (Figure 7).

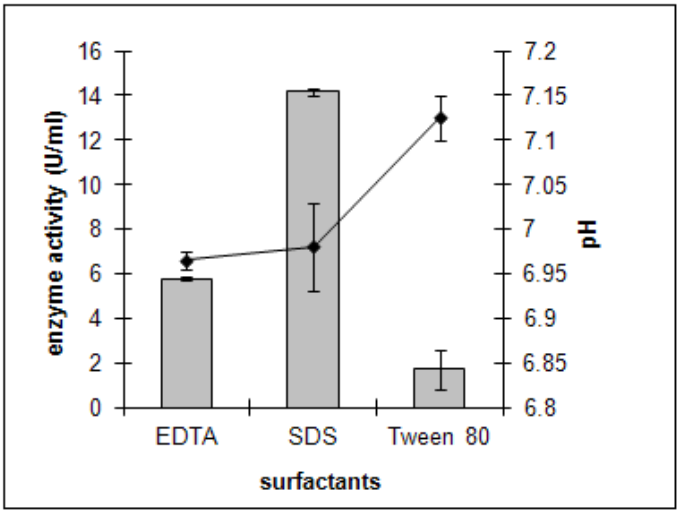

Figure 7: Effect of surfactants on phytase production

The effect of phosphorous sources on phytase production was investigated. When potassium phosphate (KH_2PO_4) was added to the medium at the concentration of 0.05 %, phytase activity was very low (data not shown). Medium with 1.5 % sodium phytate showed maximum activity (60.25 U/ml). However phytase activity decreased with the increase in the concentration of sodium phytate concentration at above 2%. After 72 hrs cultivation, 3% sodium phytate conditions, inorganic phosphate concentration in broth was determined to be 8.5% which was enough to

repress the phytase production. Phytate at lower concentration stimulates the phytase synthesis, but some phosphate liberated from phytate by phytase during cultivation significantly inhibits phytase production at high phytate concentration. Therefore, to minimize the repression by phosphate, 1.5% of sodium phytate was suitable for phytase production (figure 8). Although the presence of inorganic phosphorus is an essential ingredient of phytase production medium [20], increasing the concentration of inorganic phosphorus shows adverse effect on phytase production [21, 22]. High levels of inorganic phosphorus repress the biosynthesis of phytase [23, 24].

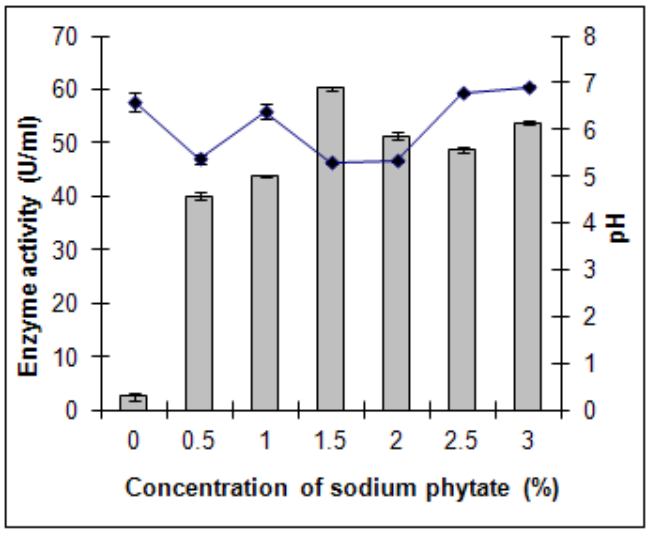

Figure 8: Effect of sodium phytate on phytase.

Based on the optimization results with the optimized parameters, an attempt was made to evaluate the extent of improvement in the phytase production. The period between 48- 72 hrs is maximum for phytase production in unoptimized condition. On optimization, maximum phytase activity of 98.76 U/ ml was achieved within 24 hrs (Figure 9, 10).

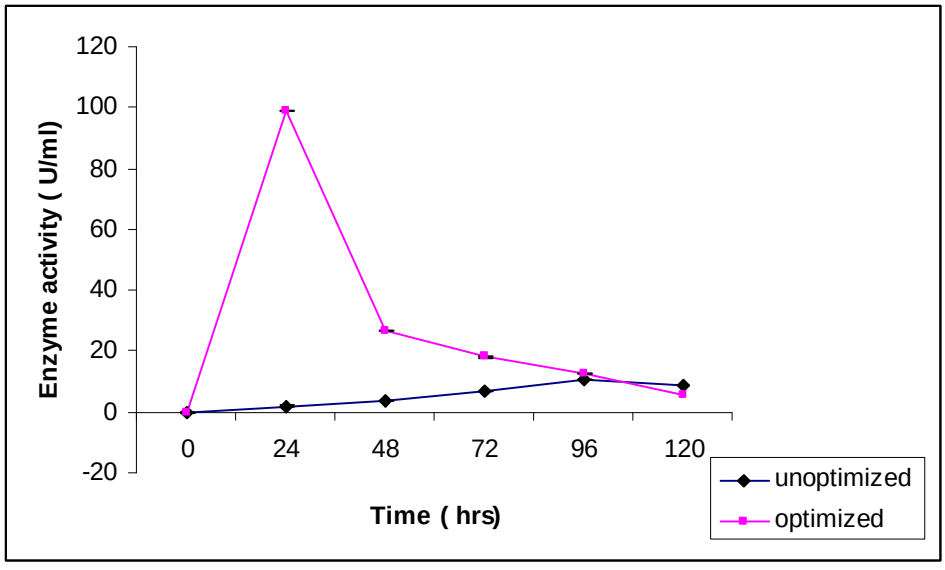

Figure 9: Time Course of phytase production in optimized and unoptimized conditions.

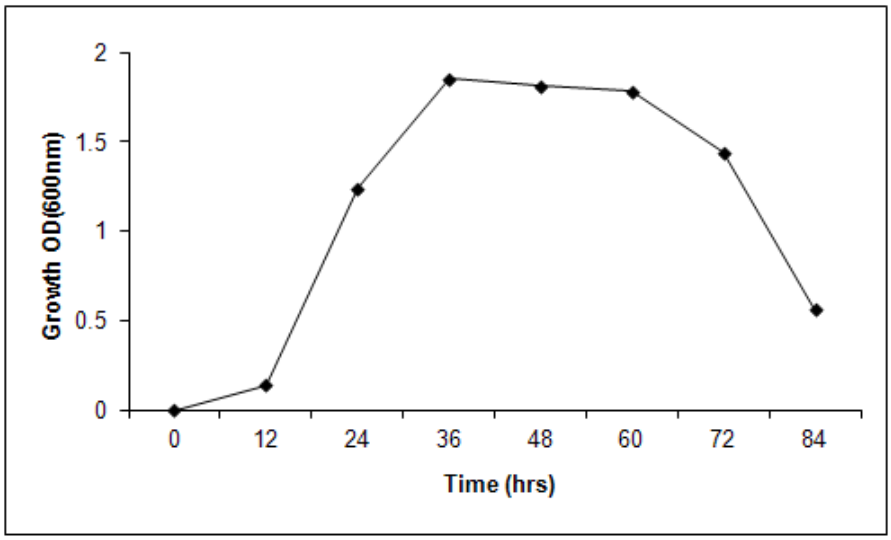

Figure 10: Growth curve of *Pseudomonas* spp in phytase specific medium.

Partial purification of phytase

Partial purification of phytase enzyme was performed by ammonium sulphate precipitation followed by dialysis. Partially purified phytase exhibited specific activity of 70.77 U/ml/mg which corresponds to 2.22 purification fold and 52.44 % Yield (Table 3).

Table3: Partial purification of phytase from *Pseudomonas* spp

Steps	Enzyme activity (U/ml)	Protein activity (mg/ml)	Specific activity (U/ml/mg)	Purification (fold)	Recovery (%)
Crude extract	2.294	0.072	31.86	1	100
Ammonium precipitation (0- 60%)	1.4787	0.040	36.96	1.16	64.45
Dialysis	1.203	0.017	70.77	2.22	52.44

CONCLUSION

This study reports biological production of phytase by *Pseudomonas* spp. Enzyme production was find maximum in presence of glucose as carbon source, yeast extract as nitrogen source, temperature 37°C and pH 6.0.Enzyme was able to tolerate broad temperature and pH range of 37° to 50° C; 4 to 10. Phytase production was doubled due to optimization. The specific activity of the crude enzyme was 31.86Umg^{-1} protein and this was increased to 70.77Umg^{-1}protein by $(NH_4)_2SO_4$ precipitation. The enzyme being thermostable and acid stable, can find application in animal feed industry for improving nutritional status of the feed and combating environmental phosphorus pollution

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