

ISOLATION OF PHYTASE PRODUCING BACTERIA FROM POULTRY FAECES AND OPTIMIZATION OF CULTURE CONDITIONS FOR ENHANCED PHYTASE PRODUCTION

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ABSTRACT

Objective: Phytase is an enzyme that catalyzes the hydrolysis of phytic acid. It is a class of phosphatase enzyme and finds use as a major animal feed supplement. In this study the effect of culture conditions for the maximum production of Phytase was assessed from a phytase producing bacteria which is screened and isolated from poultry faeces.

Methods: After the initial screening procedure, it was found that three isolates were able to hydrolyze sodium phytate. Batch production studies were carried out by varying carbon, nitrogen and phosphate sources and varying physical conditions of temperature and pH.

Results: Amongst those three isolates, *Pseudomonas fluorescens* had very high phytate degrading activity. Wheat bran, Yeast extracts and potassium dihydrogen phosphate at pH 6 was identified as the better carbon, nitrogen and phosphate sources respectively. The enzyme was stable with CaCl₂ at temperature 40-50°C and pH 6-7. Scaled up production was carried out in a 3L bioreactor and the enzyme activity found to be 32.94 U/ml. The phytase enzyme was partially purified by ammonium sulphate precipitation at 50% saturation resulting in a yield of 48.6%. Further purification was carried out using Gel filtration chromatography with a yield of 26.77%.

Conclusion: The results show that the maximum production of phytase is from *Pseudomonas fluorescens* in a 3L fermenter at pH-6, 72 hours, using wheat bran, yeast extract, potassium dihydrogen phosphate as carbon, nitrogen and phosphate sources respectively and the enzyme activity was found to be 32.94U/ml at the optimized conditions.

Keywords: Phytase, Wheat bran, Production, *Pseudomonas fluorescens* and Screening.

INTRODUCTION

In animal feeds such as cereals and legumes, phosphorous is present in the form of phytic acid. However, it is not readily assimilated by animals [1] due to the presence of inherent phytases in insufficient amounts which is required to hydrolyze the phytic acid complexes in feed [2]. Consequently, this leads to the release of undigested phytate phosphorus in faeces and urine causing severe pollution of water resources [3]. Moreover phytic acid has antinutritive properties, as it forms complexes with proteins and nutritionally important metals such as calcium, zinc, magnesium and iron decreasing their bioavailability [4]. Phytic acid is also known to inhibit a number of nutritionally important enzymes *in vivo* [2]. It is to be noted that phytic acid, owing to its strong chelating ability forms cation-phytic acid complexes with multivalent cations [5].

In order to enhance the phosphate assimilation ability of simple-stomached animal, phytases were added in animal feed [6]. Phytases can be derived from a number of sources including plants, animal tissues and microorganisms [7,8,9]. In today's world, the use of microorganisms for manufacturing products like antimicrobials [10], enzymes [11], feed, chemicals, polymers et cetera has become widespread. Recent research points towards microorganisms as a promising source for commercial phytase production [12]. Several screening programs have been carried out aiming at the isolation of different species of bacteria, yeast and fungi having extra-cellular phytase activity [13]. Phytases have been detected in various bacteria, such as *Bacillus sp* [14,15,16, 17], *Pseudomonas sp.* [18,19], *Escherichia coli* [20,21], *Enterobacter* [22], *Klebsiella sp.* [23], *Lactobacillus sanfranciscensis* and anaerobic rumen bacteria, particularly in *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella sp.*, *Mitsuokella multiacidus* and *Mitsuokella jalaludinii*. The present investigation involves isolation of phytase producing bacteria from poultry faeces and optimization of culture conditions for enhanced phytase production by *Pseudomonas fluorescens* in submerged culture conditions.

MATERIALS AND METHODS

Sodium phytate was purchased from Sigma-Aldrich (USA). All dehydrated media were procured from Himedia India Pvt Ltd

(Mumbai, India). All reagents were of analytical grade and were purchased from Qualigens (Mumbai, India). Agricultural residues such as wheat bran, Rice bran and oats were purchased from a local market in Thanjavur, India.

Isolation of Phytase-producing bacteria

Isolation of the phytase producing bacteria was carried out by sampling soil from a poultry farm at Thanjavur, Tamilnadu, India. Sediment samples (0-5 cm depth) were collected using a sterile stainless steel spatula into a sterile bottle. Three replicate samples were randomly collected from three sites (1m apart) to make a composite sample and this was used for bacterial screening. The soil samples were serially diluted with sterile deionized water and cultured on LB medium (yeast extract 5g/L, peptone 10g/L, NaCl 10g/L, Agar 20g/L). The samples were cultured at 28°C to screen isolates producing phytase extracellularly.

Cultivation of Phytase producing bacteria

The screened bacterial cultures were grown in Bushnell Haas broth (0.2 g/L MgSO₄, 0.02g/L CaCl₂, 1g/L KH₂PO₄, 1g/L K₂PO₄, 1g/L NH₄NO₃, 0.05g/L FeCl₂) using sodium phytate as the substrate. They were further grown in PSM (Phytase specific medium : 15 g/L Glucose, 5.0 g/L NH₄NO₃, 0.5 g/L KCl, 0.5 g/L MgSO₄.7H₂O, 0.01 g/L FeSO₄.7H₂O, 0.01 g/L MnSO₄.7H₂O, 0.05 g/L sodium phytate, 20.0 g/L Agar; pH adjusted to 7) at 28°C for 72 hrs. When individual isolates from LB were seeded into the PSM plate, any developing colony which produced a clear zone was considered as potential phytase producer. 100ml of sterile production medium in a 250 ml conical flask was inoculated with a 12 hour old culture grown on LB medium at 2% (v/v) and incubated under shaking conditions (180 rpm) at 28 °C for 24h. The fermented broth was clarified by centrifugation at 3,000g for 10 min and assayed for phytase activity. The selected cultures were maintained on LB agar medium and stored at 4 °C.

Identification of bacterial strains

Bacterial strains were subjected to a series of tests such as gram staining, motility test and biochemical tests for identification.

Phytase production under shake flask conditions

Phytase production by *P.fluorescens* was studied in shake flask cultures. The effect of different production parameters, i.e. simple and complex carbon sources, nitrogen sources, inorganic phosphate sources, pH on enzyme production were studied. Stability of the enzyme at different pH and temperature was also studied.

Effect of simple and complex carbon sources on phytase enzyme production

The effect of different carbon sources i.e., simple sugars like glucose, galactose, maltose, fructose, lactose, sucrose and complex sugars such as oats, wheat bran, rice bran and starch on the production of phytase were studied by replacing one carbon source with another. Conical flask with 100 ml phytase specific media were inoculated with actively growing culture and incubated at 28°C under shaking condition at 180rpm for 5 days at pH 7.

Effect of nitrogen sources on phytase enzyme production

The effect of different nitrogen sources such as ammonium sulphate, urea, peptone yeast extract, potassium nitrate and malt extract on phytase production was studied by replacing 0.5% ammonium nitrate in phytase specific medium. Conical flasks with 100ml medium were inoculated with actively growing culture and incubated at 28°C at 180rpm for 5 days at pH 7.

Effect of inorganic phosphates on phytase enzyme production

The effect of various inorganic phosphates such as disodium dihydrogen phosphate and potassium dihydrogen phosphate was studied. Conical flask with 100 ml medium were inoculated with actively growing culture and incubated at 28°C at 180rpm for 5 days at pH 7.

Effect of pH on phytase enzyme production

The effect of pH of the medium containing wheat bran (Best carbon source) on the production of phytase was studied between pH 4-9. pH was adjusted in production media by adding 0.1M NaOH/ 0.1M HCl. The flask were inoculated with actively growing culture and incubated at 28°C under at 180rpm for 5 days.

Stability of enzyme at different pH and temperature

Stability of the enzyme was studied at different pH and temperature by pre-incubation (with or without 5mM CaCl₂). The enzyme was maintained at different pH 4 to 9 (using 0.1 N NaOH and 0.1 N HCl) and different temperatures from 30 to 90°C in water bath for 30 min. The residual phytase activity in the samples at different intervals was assayed using sodium phytate as substrate.

Production of phytase in stirred tank bioreactor

After optimizing the production of phytase under shake flask conditions, it was further scaled up in 3L fermenter with working volume of 1L. The medium and the culture conditions were maintained same as that of shake flask studies. Medium containing 1.5% wheat bran, 0.5% yeast extract and supplemented with inorganic salts were used. The fermenter was run with aeration of 1vvm and agitation 250rpm. Inoculum was prepared by inoculating conical flask containing 50ml of medium incubated at 30°C at 250rpm for 24h. The fermenter containing 1L medium was in-situ sterilized and then inoculated aseptically. The fermenter was equipped with different controls such as pH, temperature, dissolved oxygen, agitation and antifoam. Samples were withdrawn at regular intervals and analyzed for protein content and phytase activity.

Estimation of protein content

Protein concentration of the sample was estimated by Lowry's method.

Phytase enzyme assay

Culture broth was centrifuged at 3000g for 15 min and the supernatant was used for assay. The phytase activity was determined by calculating the amount of liberated inorganic phosphate. The reaction mixture consist of 1ml acetate buffer (0.2M

acetate buffer pH-5.5 containing 6.82mM sodium phytate), 0.2 ml of 100 mM MgSO₄ and 0.2 ml of enzyme supernatant. It was incubated for 30min at 50°C followed by stopping the reaction by adding 1ml of 10% trichloroacetic acid. Then 1ml of Taussky shorr color reagent solution (Freshly prepared) was added followed by absorbance measurement at 660 nm using UV spectrophotometer.

Preparation of Taussky Shorr color reagent

Ammonium heptamolybdate stock solution: Dissolve 7.5gm of ammonium heptamolybdate (N₆H₂₄Mo₇O₂₄.4H₂O) in 400ml distilled water. Slowly add 22ml of 98% sulfuric acid and dilute to 500ml with water. The solution may be kept at 4°C shielded from light for 1 month.

Ferrous sulphate stock solution: 2.7 gm of ferrous sulphate in 100ml of distilled water. This solution may be kept at 4°C and shielded from light for 1 month. Ferrous sulphate solution is added to ammonium heptamolybdate solution in 1:4 ratio. This solution is freshly prepared. One unit of Phytase activity was defined as the amount of enzyme required to liberate 1 mole of phosphate per min under assay conditions. A standard graph was plotted using potassium dihydrogen phosphate with working concentration ranging from 0.1 to 0.5g/L. The specific activity was defined as the U per mg of protein. Protein concentration in the enzyme preparation during the purification steps was determined by the Lowry's method.

Enzyme purification

The culture was grown under optimized conditions and the enzyme extract was recovered by centrifugation (10,000g, 15 min).

Ammonium Sulphate Precipitation

The clear supernatant from enzyme extraction was used for ammonium sulphate precipitation at 40-80% saturation at 4°C. The protein obtained was dialyzed against 0.2 M sodium acetate buffer at pH 5.0. The precipitate formed was removed by centrifugation at 10,000 g and 4°C for 30 min and the sample enzyme activity and protein concentration was calibrated.

Gel filtration chromatography

The partially purified sample was loaded on to Sephadex column equilibrated with sodium acetate buffer (0.2 M, pH 5.0). The column was eluted first with the sodium acetate buffer followed by linear gradient of 0-0.5 M NaCl in acetate buffer (0.2M, pH 5.0) at a flow rate of 3 ml/min at 25Mpa. The bound phytase was eluted using NaCl gradient buffer.

RESULTS

Three phytase producing microbial strains were isolated from poultry faeces. Among them, *Pseudomonas fluorescens* had very high phytate degrading activity. The effect of different culture conditions like varying carbon, nitrogen, phosphate sources, temperature and pH for the maximum production of enzyme was studied. The production of phytase from *Pseudomonas fluorescens* was scaled up in a 3L stirred tank bioreactor with a working volume of 1L. This study throws some light on the large scale production of Phytase producing bacteria that will find wide commercial applications such as an animal feed additive et cetera. The data provided will provide a strong framework for further fundamental microbiological research in area of enhanced phytase production and will have tremendous significance in terms of production efficiency in the bioprocess industry.

Isolation of phytase producing bacteria

Different microbial colonies were identified and subcultured in LB media. The colonies thus sub cultured were grown on phytase specific media with sodium phytate as the substrate. By plate screening, *P. fluorescens* was found to be the best strain and could produce clear zone around the colonies (figure 1). Quantitative screening by phytase assay also showed that *P. fluorescens* to be the best strain (figure 2). *P. fluorescens* produces maximum amount of phytase (12.85U/ml).

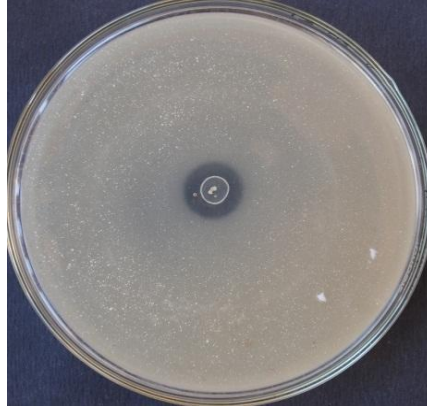


Fig. 1: *P. fluorescens* forming peripheral clear zone on phytase specific agar medium.

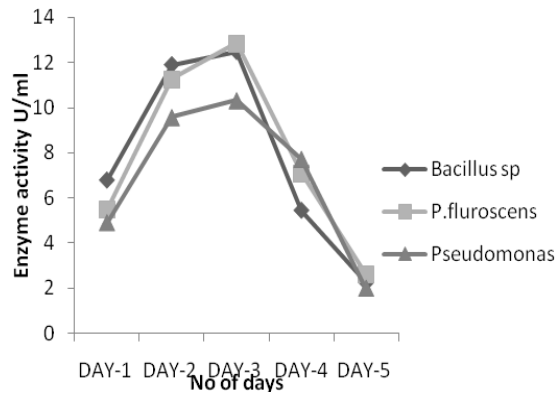


Fig. 2: Quantitative estimation of phytase producing bacterial strains

Effect of simple and complex carbon sources

Of the various carbon sources (Simple and complex), wheat bran was found to produce maximum phytase production (21.9 U/mL) (figure 3) and this is due to the high phytate content in the aleurone

layer of these grains. Comparing with all other carbon sources, lactose and galactose showed low phytase activity. Earlier report suggests that wheat bran was suitable source for the phytase production by *B.amyloliquefaciens* DS11 [14], *B.subtilis* [14] and *B.amyloliquefaciens* FZB45 [24].

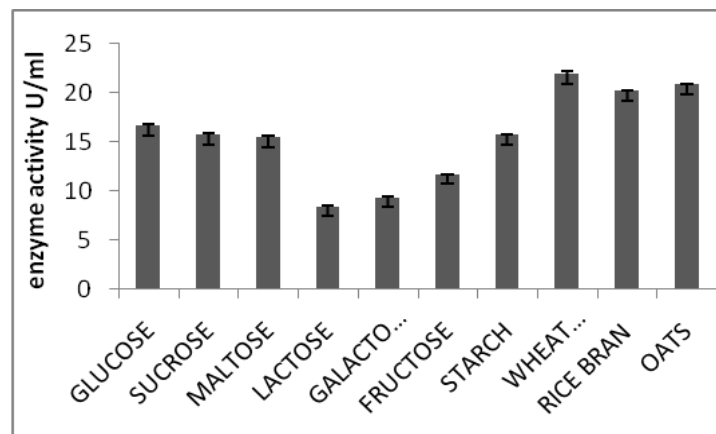


Fig. 3: Effect of simple and complex carbon sources on phytase production by *Pseudomonas fluorescens*

Effect of nitrogen sources

Of the various nitrogen sources used, yeast extract showed maximum phytase activity with wheat bran as carbon source (22.46 U/mL) (figure 4). The result of the present study showed that yeast extract with wheat bran not only increases the growth of the organism but also stimulate the phytase production in *P.fluorescens*. Next to the yeast extract, the maximum phytase activity was with peptone and ammonium sulphate. Earlier reports suggest that peptone as nitrogen source gave maximum yield in the case of *Aspergillus niger* NCIM 563 [25]. $\text{NH}_4\text{H}_2\text{PO}_4$ showed maximum yield

in *B.leavolacticus* [26] and Ammonium sulphate showed maximum yield in *Saccharomyces cerevisiae* [27].

Effect of inorganic phosphate on enzyme production

Addition of inorganic phosphates sources in fermentation medium at 0.2g/L concentration increased the phytase activity. Out of the two phosphate sources, KH_2PO_4 showed maximum phytase activity (25.90 U/mL) (figure 5) with wheat bran and yeast extract as the carbon and nitrogen sources. Generally phosphate concentration has no effect on the biomass and protein production of the organism.

The production of inorganic phosphorus is an essential ingredient of phytase production medium [28]; however it negatively regulates the phytase expression when added at concentration above 50mg/L [14]. The observed ability of *P.fluorescens* to tolerate high levels of phosphate was similarly observed in phytase producing rumen bacteria *Mitsuokella jalaudinii* [29].

Effect of Different pH

The phytase production was studied over a pH range of 5-9. pH-6 was found to be optimal for phytase production (22.82 U/mL)

(figure 6). The activity was increased at pH-6 and there was a sharp decline at pH-9.

Stability of Enzyme at Different pH and Temperature

The enzyme was pre-incubated at different temperature and pH for 30 min, with and without CaCl₂. The stability of the enzyme got reduced without CaCl₂. With CaCl₂, the activity has got stabilized (Figure 7). Present work depicts that enzyme is stable at temperatures between 40-50°C and in pH range of 6 to 7 similar to the previously observed results in *Bacillus sp* KHU-10.

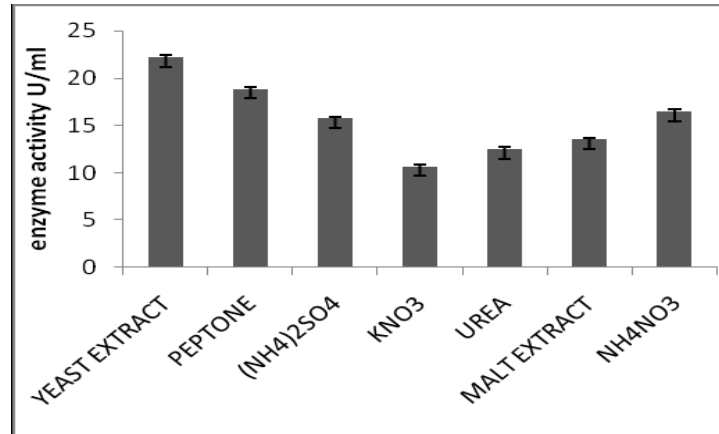


Fig. 4: Effect of nitrogen sources on phytase production by *Pseudomonas fluorescens*

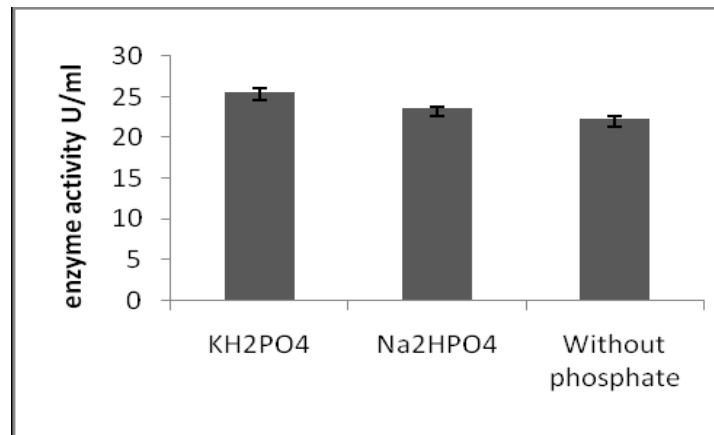


Fig. 5: Effect of inorganic phosphates on enzyme production.

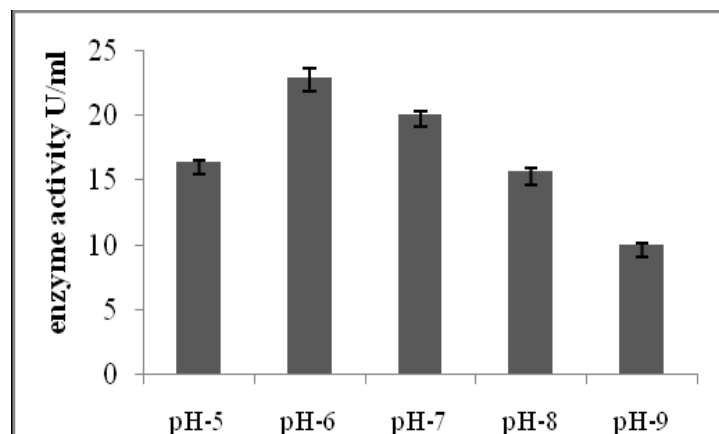


Fig. 6: Effect of pH on Phytase production

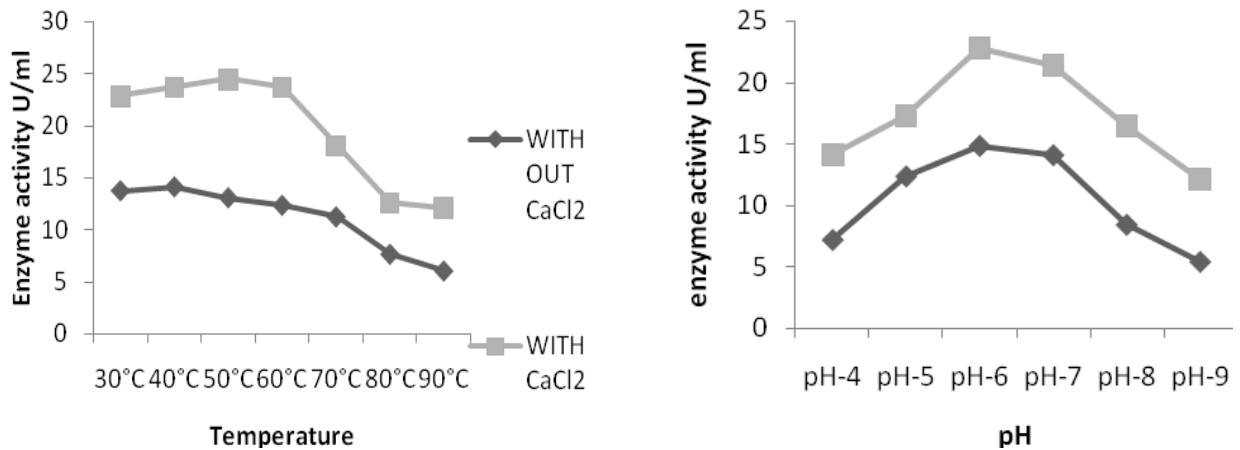


Fig. 7: Stability of enzyme at different pH and temperature.

Fermenter Studies in Stirred Tank Bioreactor

After optimizing the production of phytase under shake flask conditions, it was further scaled up to 3L fermenter with working volume 1L. The fermenter was run with aeration of 1vvm and agitation 250 rpm. Samples were withdrawn at regular intervals and analyzed for protein content and phytase activity. The pH of the fermenter was set around 6. Optimizing the pH in shake flask condition significantly increased the phytase production. In the present study, at pH-6 the maximum amount of phytase activity was observed after 72 hrs, using wheat bran, yeast extract, potassium dihydrogen phosphate as carbon, nitrogen and phosphate sources respectively. The maximum phytase activity was observed as 32.94 U/ml and the maximum specific enzyme activity was found to be 25.12 U/mg (Table 1).

Purification of Crude Phytase

Ammonium Sulphate Precipitation

The clear supernatant from enzyme extraction was used for ammonium sulphate precipitation at 50% saturation at 4°C. The total enzyme activity was found to be 16.04 U/ml and the Specific enzyme activity was 32.51U/mg. Purification fold after ammonium sulphate precipitation was 1.29 fold and the percentage yield was 48.6% (Table 2).

Table 1: Optimized condition at 1vvm and 250 rpm

No. of Days	Biomass concentration	Protein concentration mg/ml	Specific Enzyme Activity U/mg	Enzyme Activity U/ml
1	0.22	0.813	23.21	18.93
2	0.50	1.051	24.45	27.8
3	0.57	1.311	25.12	32.94
4	0.27	0.585	21.81	12.81
5	0.13	0.344	18.84	2.41

Gel Filtration Chromatography

Gel filtration chromatography is a chromatographic method in which molecules in solution are separated by their size. DEAE Sephadex column was used and fractions were eluted based on size. DEAE Sephadex column was widely used for biomolecules. In the present study, the sample got eluted and collected out showing a sharp peak in the chromatogram. Sample collected showed the total enzyme activity as 8.82 U/ml and the specific enzyme activity was 61.29 U/mg. Purification fold after gel filtration chromatography was found to be 2.44 fold and the percentage yield was 26.77% (Table 2).

Table 2: Purification strategies for Phytase from *Pseudomonas fluorescens*

Purification Steps	Total Protein mg/ml	Total Activity U/ml	Specific Enzyme Activity U/mg	Purification Fold	Yield (%)
Crude Enzyme	1.311	32.94	25.12	1.0	100
(NH ₄) ₂ SO ₄ Precipitation	0.502	16.04	32.51	1.29	48.6
GFC	0.144	8.82	61.29	2.44	26.77

DISCUSSION

There are previous reports showing the ability of microorganisms isolated from Poultry Feces to produce phytase. But the current study has generated a set of conditions where a high yield of phytase is produced as compared to the other similar optimization experiments performed for phytase production [30]. The results shows that wheat bran was identified as the best carbon source with enzyme activity 21.9 U/ml and yeast extract along with wheat bran induced the activity to 22.46 U/ml. *Pseudomonas fluorescens* was insensitive to the inorganic phosphates and the activity increased to a great extent when KH₂PO₄ is used along with wheat bran and yeast extract to 25.90 U/ml. The optimum pH for the growth of the organism is 6. The crude enzyme was stable at 40 to 70°C for over 30 mins of incubation with 5mM CaCl₂ and also, the crude enzyme showed stability at pH 6 and pH 7 with 5mM CaCl₂. Scale up in production was done in 3L fermenter by using the shake flask parameters at 1vvm and 250 rpm. The enzyme activity was found to

be 32.94U/ml. Further the enzyme was purified by ammonium sulphate precipitation at 50% saturation and it is then further purified by Gel filtration chromatography and the yield of enzyme purified was 26.77%.

CONCLUSION

Keeping the potential applications of phytase in mind, this work merits special interest towards producing novel thermostable phytase enzyme that finds use in biotechnological applications in animal feed additives, human health, pulp and paper industries. When produced in a 3L fermenter, enzyme activity was found to be 32.94U/ml at the optimized conditions of media, temperature, pH and appropriate salt contents i.e at pH-6 the maximum amount of phytase activity was observed after 72 hrs, using wheat bran, yeast extract, potassium dihydrogen phosphate as carbon, nitrogen and phosphate sources respectively. In conclusion, the above experiments and data are the basic and fundamental steps directed

towards further research in the phytase production arena aimed at economical, environmental friendly and high efficient enzyme production.

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