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# Biosynthesis, purification, kinetic and thermodynamic analysis of phytase from *Aspergillus oryzae*

# Biosíntesis, purificación, análisis cinético y termodinámico de la fitasa de *Aspergillus oryzae*

I.U. Haq*, M.U. Javed, A. Nawaz, Y. Arshad, H. Mukhtar

*Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan.*

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## Abstract

Phytic acid is not digested by monogastric animals due to deficiency of an enzyme (Phytase) in their digestive tube. Due to this reason, the poultry industry needs to add potent phytase in its feed for faster growth rates. Therefore, phytase producing fungal strains (64) were isolated and subjected to submerged fermentation, and maximum production (21.79±0.05 U/mL/min) was obtained by fungal isolate UJIIB-29, which was identified as *Aspergillus oryzae* after18S rRNA sequencing. Under optimized conditions, best results were obtained using glucose phosphate broth as a medium after 7 days of incubation, at 30 ºC, pH 5.5 with 0.5% glucose and 0.3% ammonium sulfate as carbon and nitrogen sources, respectively when it was inoculated with 4% conidial suspension. Purification of phytase by anion exchange chromatography resulted in 89.8% yield, 1.93 folds' purification, and 53.5 U/mg specific activity. The molecular weight of purified phytase was determined as 53 kDa whereas, $K_m$ and $V_{max}$ were determined as 3.41 mM and 24.6±0.05 U/mL/min, respectively. Thermodynamic studies of the enzyme revealed activation energy (Ea) and enthalpy of activation (ΔH) as 16.84 KJ/mol and 14.32 KJ/mol, respectively. Phytase at 30ºC and pH 5.5 showed maximum stability for 6 hours but enzyme activity remained stable up to 60 ºC. The findings of the current study will help in developing an economical process for phytase biosynthesis. Besides, kinetic and thermodynamic studies will help in assessing the applicability of phytase in harsh conditions.

*Keywords*: phytic acid, submerged fermentation, optimization, catalysis, thermodynamics.

## Resumen

El ácido fítico no es digerido por animales monogástricos debido a la deficiencia de una enzima (fitasa) en su tubo digestivo. Debido a esta razón, la industria avícola necesita agregar fitasa potente en su alimentación para lograr tasas de crecimiento más rápidas. Por lo tanto, las cepas fúngicas productoras de fitasa (64) se aislaron y se sometieron a fermentación sumergida, y la producción máxima (21.79±0.05 U/ml/min) se obtuvo mediante el aislamiento fúngico UJIIB-29, que se identificó como *Aspergillus oryzae* después de la secuenciación de 18S rRNA. En condiciones optimizadas, se obtuvieron los mejores resultados utilizando caldo de fosfato de glucosa como medio después de 7 días de incubación, a 30 ° C, pH 5.5 con 0.5% de glucosa y 0.3% de sulfato de amonio como fuentes de carbono y nitrógeno, respectivamente cuando se inoculó con 4% suspensión conidial. La purificación de fitasa por cromatografía de intercambio aniónico dio como resultado un 89,8% de rendimiento, una purificación de 1,93 veces y una actividad específica de 53.5 U / mg. El peso molecular de la fitasa purificada se determinó como 53 kDa, mientras que $K_m$ y $V_{max}$ se determinaron como 3.41 mM y 24.6±0.05 U/mL/min, respectivamente. Los estudios termodinámicos de la enzima revelaron la energía de activación (Ea) y la entalpía de activación (ΔH) como 16,84 KJ / mol y 14,32 KJ / mol, respectivamente. La fitasa a 30ºC y pH 5.5 mostró una estabilidad máxima durante 6 horas, pero la actividad enzimática permaneció estable hasta 60 ºC. Los hallazgos del estudio actual ayudarán a desarrollar un proceso económico para la biosíntesis de fitasa. Además, los estudios cinéticos y termodinámicos ayudarán a evaluar la aplicabilidad de la fitasa en condiciones difíciles.

*Palabras clave*: Biosensores, biorreportadores, luxCDABE, biodisponibilidad, naftaleno, tolueno, Isopropilbenceno, hidrocarburo.

* *Corresponding author. E-mail*: dr.ikramulhaq@gcu.edu.pk
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# 1 Introduction

Phytase (E.C. 3.1.3.8) is phosphomonoesterase, which hydrolyzes phytic acid to Myo-inositol and phosphate (inorganic) (Aziz *et al*., 2015). The acid, salt, and calcium or magnesium derivatives of phosphorus are phytic acids, phytate, and phytin, respectively. Phosphorus is a major ingredient of feed and needed for the growth of animals. Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6 hexakisphosphates) is the main source of organic phosphorus in animal nutrition and principal storage form of phosphate in plants. It is widely available in legumes, cereal grains, seeds, and diet of animals and humans (Gupta *et al*., 2014). However, the molecule of phytic acid has six radicals (negatively charged) and thus show a strong ability to bind with several cations, and thus, the phytic acid molecules have a certain anti-nutritive activity. For this reason, low residual phytate content in the feed is desired (Mendoza-Avandano *et al*., 2019). Similarly, Phytates, six ringed structures, have 50-80% phosphorous in a bound form, which cannot be broken by the endogenous enzyme of the poultry (Akbarzadeh *et al*., 2015, Dersjant-Li *et al*., 2015). Phytase use as a feed additive is approved as GRAS (generally regarded as safe) in 22 countries to improve the nutritional value of feed (Rosenfelder *et al*., 2020).

The main sources of phytase are plants (rice, soybeans, fava bean, wheat, maize, oilseeds, etc.) and microbes (Wang *et al*., 2013). The common genera of fungi and bacteria that produce this enzyme are *Aspergillus, Mucor, Rhizopus*, and *Penicillium* (Cangussu *et al*., 2018), *Bacillus*, enterobacter, *Pseudomonas* and anaerobic ruminal bacteria (Kumar *et al*., 2013). Fungi are extraordinary compared to other organisms because of their capacity to secrete high amounts of enzymes appropriate for industrial applications (Aftab *et al*., 2019). Submerged fermentation is preferred for its industrial production as extraction and purification of the enzyme by this procedure is simple, economical as well as easy to manage and control (Kłosowski *et al*., 2018). Optimization is an undeniable component of any commercial goal (Shakir *et al*., 2018). The optimization of cultural conditions must be done for the maximum production of the enzyme. Some of these important factors include fermentation medium, incubation period, temperature, pH, carbon source, nitrogen source, and the size of the inoculum. For the synthesis of enzymes, explicit supplements are required, for example, magnesium, potassium, calcium, and phosphorus. These components are essential to improve and streamline the production of phytase (Tian and Yuan, 2016). Enzyme purification is of pronounced significance and the grade of purity of an enzyme depends upon its end-use. The objective behind purification is to achieve the greatest possible yield of the enzyme with the maximum catalytic activity and the ultimate potential purity (Schoene *et al*., 2016).

$K_m$ and $V_{max}$ are the two main kinetic factors that are studied while characterization of an enzyme. $V_{max}$ of an enzyme is attained when the enzyme completely binds to its substrate. $K_m$ of an enzyme is the substrate concentration at half the maximum velocity, which implies that half of the active sites of enzyme are used and the substrate is converted into the product. Enzyme with lower $K_m$ proves beneficial for industries; as a maximum reaction velocity is achieved easily when employed by a certain substrate. Enzyme kinetic analysis has moved forward to increase efficiency and cost-effectiveness for the modern industry (Reddy *et al*., 2015). The thermodynamic characteristic of an enzyme is the utilization of substrate by an enzyme with changing the temperature. The energy of activation (Ea), entropy ($\Delta S$) and enthalpy ($\Delta H$) are essential thermodynamic parameters, which are considered while analyzing the efficiency of an enzyme (Joshi and Satyanarayana, 2015).

In poultry, phytase is the most important enzyme used as a feed supplement. It hydrolyse the phytate present in the feed, which has anti-nutritional activity. Phytase, when added, increases the bioavailability of feed nutrients and decreases the price of di-calcium phosphate, which is added in poultry feed as surplus phosphorus and phosphate. The mineral intake and feed conversion ratio of animals, consuming phytate phosphorus, are increased by adding microbial phytase in the feed (Ptak *et al*., 2015). This research aims to produce phytase from indigenously isolated fungi, its production process optimization, purification, kinetic and thermodynamic analysis to provide the poultry industry with an efficient enzyme that will make poultry feed more digestible for chicks.

## 2 Materials and methods

### 2.1 Isolation and screening

The isolation of fungi was carried out by collecting 103 soil samples (associated with poultry exposure) from different cities of Pakistan (Lahore, Shahdara, Narowal, Islamabad, Faisalabad, Gujranwala, Phool Nagar, Skardu, etc.). After removing the top layer of soil, samples were collected and transported in sterile polythene bags. Phytate screening agar medium of pH 5 was used for the isolation and screening of phytase producing fungi having the composition (g/L); glucose (15g), ammonium sulfate (5g), KCl (0.5 g), magnesium sulfate heptahydrate (0.1 g), NaCl (0.2 g), $CaCl_2$ (0.2 g), ferrous sulfate heptahydrate (0.01 g), manganese sulfate (0.01g), sodium phytate (2.5 g) and agar (18 g) (Hosseinkhani and Hosseinkhani, 2009). The medium was autoclaved at 121 ºC for 15 min. After spreading the serially diluted soil suspension ($10^{-5}$ and $10^{-7}$), Petri plates were incubated for 5 to 7 days at 30 ºC. Observed fungal colonies were then streaked on Potato Dextrose Agar (PDA) slants and incubated at 30 ºC. The cultures were stored at 4 ºC for further use.

### 2.2 Enzyme production and extraction

Submerged fermentation was carried out for the production of the enzyme. The composition (g/L) of fermentation medium (Glucose phosphate broth) was glucose (10 g), ammonium sulfate (3 g), KCl (0.5 g), $MgSO_4$ (0.5 g), $CaCl_2$ (0.1 g), and $KH_2PO_4$ (3g) (Sandhya *et al*., 2015). The pH of the medium was maintained at 5.5. The isolates were cultured in a 25mL fermentation medium by inoculating 1mL of conidial suspension. Flasks were incubated at 30 ºC in a shaking incubator for 7 days. After incubation, the culture medium was filtered with the help of muslin cloth. Centrifugation (SIGMA laboratory centrifuges 3K30) was done at 6000 rpm for 10 min to remove any remaining solid debris. The enzyme activity was estimated by using supernatant as a crude enzyme (Arif *et al*., 2018).

### 2.3 Phytase assay

The inorganic phosphorus that is produced by the hydrolysis of phytic acid was detected by the colorimetric method (Gaind and Singh, 2015). To evaluate the inorganic phosphorus, a standard curve of phosphorus standard solution was plotted. The enzyme assay was performed by taking 0.5 mL magnesium sulfate solution (0.1 M), 1 mL sodium phytate substrate solution (0.2 M), and 0.5 mL enzyme. It was then placed in a water bath at 30°C for 15 minutes. Then, 1 mL of 10% TCA (Trichloroacetic acid) was added to stop the reaction. The absorbance using spectrophotometer was taken at 660nm, after the addition of 1 mL of coloring agent i.e. Tausky Shorr Coloring Reagent (TSCR) (Dobre *et al*., 2015).

"One unit of phytase enzyme is defined as the amount of enzyme which is needed to release the 1.0 $\mu$mole of inorganic phosphorus per minute under the standard conditions for an enzyme assay" (Rachmawati *et al*., 2017).

$$\text{Phytase activity (U/mL/min)} = \frac{\mu\text{M of phoshorous released} \times \text{Dilution factor} \times (5)}{(2) \times \text{Incubation time (min)} \times (0.5)} \quad (1)$$

Where 5 is the volume of total reaction mixture; 2 is the volume of taken from total reaction mixture; and 0.5 is the volume of enzyme. All these values are in mL.

### 2.4 Protein estimation

The estimation of total protein was carried out by Bradford's method. The standard protein used as Bovine Serum Albumin (BSA) (Bradford, 1976).

### 2.5 Identification

Molecular identification of organism at genus level was carried out by using 18S rRNA (Sequence obtained by using commercial service of Macrogen, Korea), firstly BLAST was done, then related sequences were obtained, and further clustal W was used for checking the alignment of the sequence of interest with known sequences. A phylogenetic tree was constructed to check the evolutionary relationship (Gupta *et al*., 2014).

### 2.6 Optimization of cultural conditions

Various conditions for fermentation culture were optimized to check the effect of an individual variable on the activity of the enzyme. The parameters optimized were different media (Phytate specific broth, phytate specific medium, glucose phosphate broth, and potato dextrose broth), the incubation period (3-9 days), temperature (25-60 ºC), pH of

media (4-8), carbon sources (Lactose, soluble starch, cellulose, sucrose, glucose, and fructose), organic and inorganic nitrogen sources (peptone, ammonium acetate, urea, yeast extract, tryptone, potassium nitrate, beef extract, sodium nitrate, ammonium chloride, and ammonium sulfate) and inoculum size (1-5%) (Ajith *et al*., 2018).

### 2.7 *Purification*

Fractional precipitation was carried out using different concentrations of ammonium sulfate (10-90%) for partial purification of the protein of interest and dialyzed to remove excessive salt (Onem and Nadaroglu, 2014). To further purify the enzyme, anion-exchange chromatography (Bio-Rad BioLogic LP, USA) was done by utilizing the Bio-scale Mini MacroPrep DEAE cartridge column using 50 mM Tris-Cl (pH 8) as an equilibrating buffer and 50 mM Tris-Cl with 0.1 M NaCl as elution buffer. The fractions obtained were analyzed by SDS- PAGE for purity (Demir *et al*., 2017).

### 2.8 *Characterization of enzyme*

Different parameters for the characterization of the enzyme were studied to obtain optimized conditions of enzyme activity. Parameters studied for this purpose included temperature (30-60°C), temperature stability, pH of an enzyme using sodium acetate (4.5-6), and Tris-Cl (6.5-7.5) buffers, pH stability and substrate concentration (0.1-1%).

### 2.9 *Statistical analysis*

All the experiments were conducted in triplicates. Statistical analysis of results was determined by computer statistical software. The probability ($p$) indicated in the form of Duncan's multiple range tests, showed significant differences among the triplicates (Duncan, 1955).

### 2.10 *Materials*

All the chemicals used during the research were bought from authentic companies such as Uni chem (India), Sigma-Aldrich (USA), Fischer scientific (USA), Merck (Germany), and Acros Ltd. (USA).

## 3 Results and discussion

### 3.1 *Isolation/ Primary screening*

For the isolation of the fungi having the ability to produce phytase, various soil samples were collected and spread aseptically on a plate having phytate screening agar medium, which allowed the growth of only phytase producing fungi. It resulted in the isolation of sixty-four different fungal strains, as shown in table 1.

Collection of soil samples for isolation of phytase producing fungi was done from the poultry manure-dumping sites. Dobre *et al*. (2015) and Liu *et al*. (2018) also preferred the similar source for isolation because phytase is used in poultry feed. Primary screening of fungi was done by using phytate screening agar (PSM) which specifically promoted the growth of phytase producing fungi. Sandhya *et al*. (2015) and Arif *et al*. (2018) applied the same kind of method for primary screening because this medium contains a salt of phytic acid, which is a specific substrate for phytase.

### 3.2 *Secondary screening*

The secondary screening was carried out using submerged fermentation. Fungal strain UJIIB-29 showed the maximum phytase activity 21.79 ± 0.05 U/mL/min after 7 days of incubation at 30°C. Estimation of phytase for all other fungal isolates is given in Table 1. Gunashree and Venkateswaran, (2015) worked on *Aspergillus niger* CFR 335 (32.6 ± 3.1 U/mg) and Yasser *et al*., (2018) also used the similar protocol for screening of *Aspergillus tubingensis*) for phytase production (20.56 IU/g).

### 3.3 *Molecular identification*

Macrogen sequencing company carried out the molecular identification based on 18S rRNA. The company provided sequence and further BLAST and Clustal W were used to identify the strain that was most closely related to *Aspergillus oryzae* RIB40 (XR_002735719.1). The phylogenetic tree constructed using these results is shown in figure 1. A similar technique for the identification of *Aspergillus niger, Aspergillus fumigatus, Aspergillus awamori*, and *Aspergillus terreus* was used by Ahmad *et al*. (2016). Tsai *et al*., (2019) also identified *Aspergillus fumigatus* and *Aspergillus terreus* by 18S rRNA

technique. The gene sequence ia provided below:

>190412-038_G13_U1_NS8.ab1 1279
CCCTATGACCGGGTTTGACAACTTTCCGGCCCTGGGGGGTCGTTGCCAAC
CCTCCTGGGCCAGTCCGAAGGCCTCACCGAGCCATTCAATCGGTAGTAGC
GACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCGGCACGAGCTGATGA
CTCGTGCCTACTAGGCATTCCTCGTTGAAGAGCAATAATTGCAATGCTCT
ATCCCCAGCACGACAGGGTTTAACAAGATTACCCGGACCTCTCGGCCAAG
GTGATGTACTCGCTGGCCCTGTCAGTGTAGCGCGCGTGCGGCCCAGAACA
TCTAAGGGCATCACAGACCTGTTATTGCCGCGCACTTCCATCGGCTTGAG
CCGATAGTCCCCCTAAGAAGCCAGCGGCCCGCAAACGCGGACCGGGCTAT
TTAAGGGCCGAGGTCTCGTTCGTTATCGCAATTAAGCAGACAAATCACTC
CACCAACTAAGAACGGCCATGCACCACCATCCAAAAGATCAAGAAAGAGC
TCTCAATCTGTCAATCCTTATTTTGTCTGGACCTGGTGAGTTTCCCCGTG
TTGAGTCAAATTAAGCCGCAGGCTCCACGCCTTGTGGTGCCCTTCCGTCA
ATTTCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAGAACCCAAAAA
CTTTGATTTCTCGTAAGGTGCCGAGCGGGTCATCATAGAAACACCGCCCG
ATCCCTAGTCGGCATAGTTTATGGTTAAGACTACGACGGTATCTGATCGT
CTTCGATCCCCTAACTTTCGTTCCCTGATTAATGAAAACATCCTTGGCGA
ATGCTTTCGCAGTAGTTAGTCTTCAGCAAATCCAAGAATTTCACCTCTGA
CAGCTGAATACTGACGCCCCCGACTATCCCTATTAATCATTACGGCGGTC
CTAGAAACCAACAAAATAGAACCGCACGTCCTATTCTATTATTCCATGCT
AATGTATTCGAGCAAAGGCCTGCTTTGAACACTCTAATTTTTTCACAGTA
AAAGTCCTGGTTCCCCCCACAGCCAGTGAAGGCCATGAGGTTCCCCAGAA
GGAAAGGTCCAGCCGGACCAGTACTCGCGGTGAGGCGGACCGGCCAGCCA
GACCCAAGGTTCAACTACGAGCTTTTTAACTGCAACAACTTTAATATACG
CTATTGGAGCTGGAATTACCGCGGCTGCTGGCCCCAGACTTGCCCTCCAA
TTGTTCCTCGTTAAGGGATTTAGATGGACTCTTTCCATTTCGAGAACCCA
AAAGAACCCCGAATCCAGATTTTTTGGCC

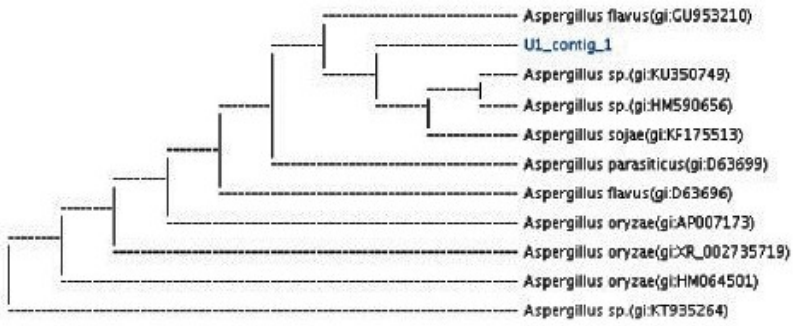



Fig. 1. Phylogenetic tree showing the evolutionary relationship for fungal isolate UJIIB-29.

## *3.4 Optimization of cultural conditions*

### *3.4.1 Media*

Four different media were assessed for their effect on the production of phytase. It can be seen in figure 2(a) that all the media supported the production of an enzyme, but among them, glucose phosphate broth (22.3±0.30 U/mL/min) was selected as the best production medium. The increased activity in glucose phosphate broth is maybe because it has a mixture of several macro and micronutrients that are essential for the growth of fungi. Moreover, the presence of potassium may have assisted the production of phytase by regulating pH through $K^+/H^+$ transport channels of the membrane. $Mg^+$ ions interact with membrane and reduce the permeability of anions through the

membrane. Further, the divalent salts in the medium may have acted as a co-factor of an enzyme (Kim *et al*., 2007). The results reported by Shah *et al*. (2017) contradicted the present finding and showed that *Aspergillus niger* NCIM 563 gave the best result with basal media. This contradiction might be due to the use of different *Aspergillus* spp. However, Kim *et al*. (2007) reported similar activity of *Saccharomyces cerevisiae* (20 U/mL/min) on glucose phosphate broth.

Table 1. Isolation of phytase producing fungi using phytase screening agar medium and estimation of phytase production by submerged fermentation.

| Location | Phytase production | Activity (Units/ml/min) | Strain |
|---|---|---|---|
| Noonar (Narowal) | + | 13.2±0.25 | UJIIB-1 |
| Ferozepur (Narowal) | + | 9.78±0.25 | UJIIB-2 |
| Kartarpur (Narowal) | + | 12.65±0.25 | UJIIB-3 |
| Veeroki (Narowal) | + | 11.09±0.25 | UJIIB-4 |
| Nadra baad (Lahore) | + | 16.98±0.25 | UJIIB-5 |
| Balakot | + | 13.76±0.25 | UJIIB-6 |
| Waqas market (Lahore) | + | 8.56±0.25 | UJIIB-7 |
| Biological garden (GCU) | + | 15.34±0.25 | UJIIB-8 |
| Poultry farm I (Phoolnagr) | + | 12.12±0.25 | UJIIB-9 |
| Poultryfarm II (Phoolnagr) | + | 17.9±0.25 | UJIIB-10 |
| Poultryfarm IV (Phoolnagr) | + | 18.32±0.25 | UJIIB-11 |
| Pasroor | + | 4.67±0.25 | UJIIB-12 |
| Wazirabad | + | 9.45±0.25 | UJIIB-13 |
| Poultry farm VII (Pholngr) | + | 13.39±0.25 | UJIIB-14 |
| Dairy farm I (Phoolnagr) | + | 17.21±0.25 | UJIIB-15 |
| Dairy farm III (Phoolnagr) | + | 19.65±0.25 | UJIIB-16 |
| Dairy farm IV (Phoolnagr) | + | 18.88±0.25 | UJIIB-17 |
| Dairy farm V (Phoolnagr) | + | 2.89±0.25 | UJIIB-18 |
| Dairy farm VI (Phoolnagr) | + | 11.74±0.25 | UJIIB-19 |
| Manga mandi | + | 10.3±0.25 | UJIIB-20 |
| Ichra (Lahore) | + | 9.54±0.25 | UJIIB-21 |
| Dhamthal | + | 18.94±0.25 | UJIIB-22 |
| Zafarwal | + | 10.9±0.25 | UJIIB-23 |
| Patoki | + | 9.12±0.25 | UJIIB-24 |
| Kasur (Salamat pura) | + | 14.54±0.25 | UJIIB-25 |
| Kasur (katchery road) | + | 12.67±0.25 | UJIIB-26 |
| Canal bank housing scheme | + | 8.54±0.25 | UJIIB-27 |
| FBIC (Lahore) | + | 11.09±0.25 | UJIIB-28 |
| Shahdara (Lahore) | + | 21.79±0.25 | UJIIB-29 |
| Plants Nursery (Lahore) | + | 18.32±0.25 | UJIIB-30 |
| Cantt (Islamabad) | + | 9.31±0.25 | UJIIB-31 |
| Garden town (Lahore) | + | 15.96±0.25 | UJIIB-32 |
| Okara (garden) | + | 20.93±0.25 | UJIIB-34 |
| Basirpur | + | 16.32±0.25 | UJIIB-35 |
| Gujranwala | + | 18.21±0.25 | UJIIB-36 |
| Nankana sahib | + | 19.01±0.25 | UJIIB-37 |
| Okara(field of corn) | + | 18.93±0.25 | UJIIB-38 |
| D.G.K (Taunsa Shareef) | + | 7.21±0.25 | UJIIB-39 |
| D.G.Khan (Kala kalooni) | + | 8.98±0.25 | UJIIB-40 |
| Johar town (Lahore) | + | 10.32±0.25 | UJIIB-41 |
| Sahiwal (Farid town) | + | 14.65±0.25 | UJIIB-42 |
| Qadirabad | + | 11.34±0.25 | UJIIB-43 |

| | | | |
|---|---|---|---|
| PCSIR (Lahore) | + | 6.23±0.25 | UJIIB-44 |
| Faislabad (Johar colony) | + | 1.33±0.25 | UJIIB-45 |
| Sargodha | + | 3.78±0.25 | UJIIB-46 |
| Samnabad (LHR) | + | 11.09±0.25 | UJIIB-47 |
| Mustafa Abad (Lahore) | + | 8.22±0.25 | UJIIB-48 |
| Railway station Lahore | + | 4.95±0.25 | UJIIB-49 |
| Pasrur | + | 17.23±0.25 | UJIIB-50 |
| Chawinda | + | 16.21±0.25 | UJIIB-51 |
| D.G Khan | + | 10.10±0.25 | UJIIB-52 |
| Derra Gujran Dairy farm I | + | 17.99±0.25 | UJIIB-53 |
| Derra Gujran Dairy farm II | + | 13.54±0.25 | UJIIB-54 |
| Faiq's Home I | + | 7.78±0.25 | UJIIB-55 |
| Faiq's Home II | + | 2.43±0.25 | UJIIB-56 |
| Kamra | + | 11.43±0.25 | UJIIB-57 |
| Peshawar | + | 8.67±0.25 | UJIIB-58 |
| Faislabad | + | 10.48±0.25 | UJIIB-59 |
| Sargodha | + | 7.34±0.25 | UJIIB-60 |
| Pattoki | + | 15.76±0.25 | UJIIB-61 |
| Garhi shahu (Lahore) | + | 12.59±0.25 | UJIIB-62 |
| Lidhar (Lahore) | + | 5.38±0.25 | UJIIB-63 |
| Ali park (Lahore) | + | 16.37±0.25 | UJIIB-64 |

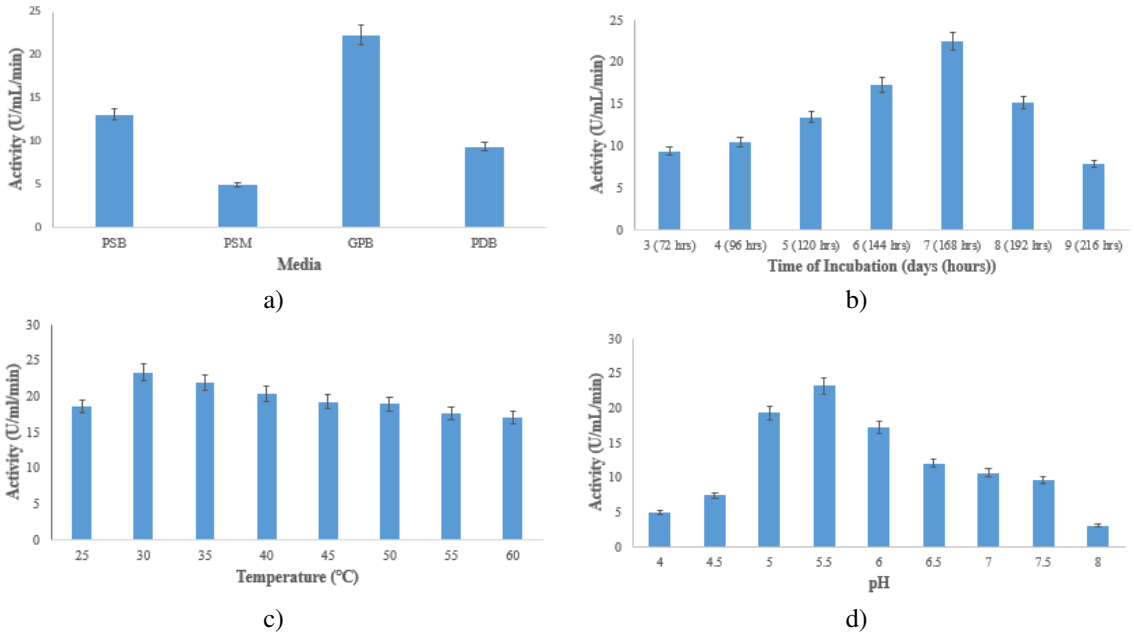



Fig. 2. Optimization of cultural conditions including: (a) effect of fermentation medium, (b) effect of incubation time, (c) effect of incubation temperature, and (d) effect of pH on enzyme production from *A. oryzae* strain (UJIIB-29). Y-error bars represents the standard deviation (SD≤ ±0.05) between three replicates.

### *3.4.2 Incubation time*

Effect of different incubation periods i.e. 3-9 days was checked for the production of phytase. It was observed that maximum activity of 22.45±0.25 U/mL/min was obtained after 7 days of incubation as shown in figure 2(b). Buddhiwant *et al*. (2016) also represented closely related results by solid-state fermentation from *Aspergillus niger* because the cells entered the log phase slowly due to the medium used could be consumed by the fungi at a slow rate. While, Tian and

Yuan (2016), obtained maximum activity of phytase from *Aspergillus ficuum* after 6 days of incubation. This could be due to the difference in metabolic rate of *Aspergillus ficuum* compared to the fungal strain used in the current study.

### *3.4.3 Temperature*

Effect of temperature (25 ºC-60 ºC) on enzyme production was checked and maximum enzyme units (23.25±0.01 U/mL/min) were obtained at 30 ºC as shown in figure 2(c). The optimum temperature was determined as 30°C, due to the mesophilic nature of fungi *A. oryzae*. The temperature was directly related to the metabolic needs of an organism so it affects the growth and formation of products by microorganisms. At high temperatures, proteins get denatured and result in the death of microorganisms. Ajith *et al*. (2018) reported similar results from *Aspergillus foetidus* MTCC 11682 (52.7 FTU/mL). Qasim *et al*. (2017), also supported our result by showing 30°C as the optimum temperature for *Aspergillus tubingensis* SKA.

### *3.4.4 pH*

The optimal pH for the fermentation medium to produce phytase by *Aspergillus oryzae* was analyzed by varying the pH (4-8) of the medium. The maximal activity of enzyme 23.75±0.02 U/mL/min was obtained using a medium having pH 5.5 and minimal activity was obtained at pH 8 (3.08±0.02 U/mL/min) as shown in figure 2(d).

Optimization of pH depicted that fungal strain (UJIIB-29) producing phytase grow at slightly acidic pH 5.5 (23.75±0.02 U/mL/min). The comparison of research and results of Qasim *et al*. (2017), using *Aspergillus tubingensis* SKA (pH 5) and Mezeli *et al*. (2017) who obtained maximum activity at pH 4.5-5.5 by *Aspergillus niger* showed that strains were isolated from acidic environment that is why they gave optimal results at acidic pH.

### *3.4.5 Carbon source*

Six different carbon sources i.e. lactose, soluble starch, cellulose, sucrose, fructose, and glucose were substituted in the fermentation medium to analyze their effect on the phytase production (figure 3a). It was recorded that better phytase activity (23.88±0.25 U/mL/min) was gained by glucose as a carbon source.

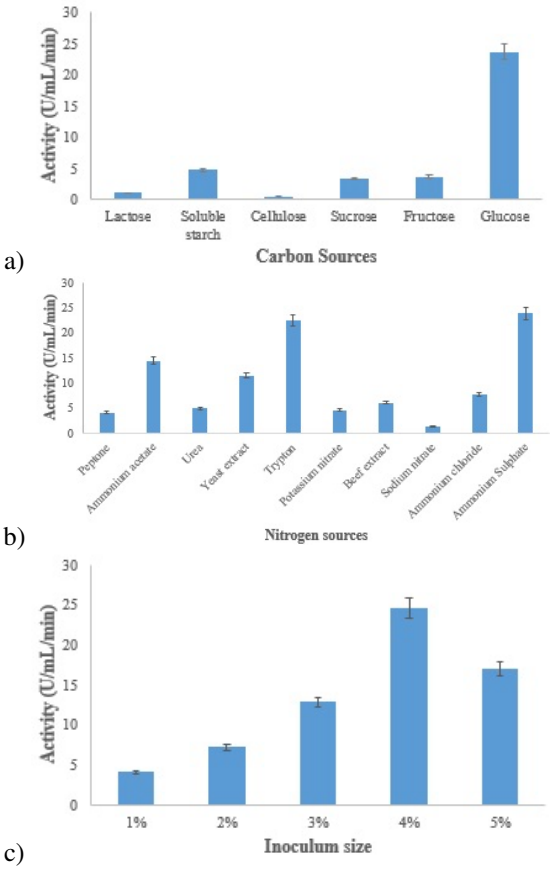



Fig. 3. Optimization of cultural conditions including: (a) effect of different carbon sources, (b) effect of different nitrogen sources, and (c) effect of inoculum size on the enzyme production from *A. oryzae* strain (UJIIB-29). Y-error bars represents the standard deviation (SD≤ ±0.05) between three replicates.

Glucose is an easily metabolizable carbon source that provides enough energy for the growth of *A. oryzae* compared to other more difficultly utilizable carbon sources. Kanti *et al*. (2019) reported similar results from *A. niger* Str3 because it assisted in fast growth than other sugars and it was used up first in sugar mixtures. Sreeja *et al*. (2019) contradicted these results, reporting Lactose as the best carbon source. This might be because *Aspergillus foetidus* MTCC 11682 (13 FTU/mL) could consume different carbon sources according to its metabolic needs.

### *3.4.6 Nitrogen source*

Different organic and inorganic nitrogen sources affecting enzyme production such as peptone, ammonium acetate, sodium nitrate, urea, beef extract, ammonium chloride, tryptone, potassium nitrate, yeast

extract, and ammonium sulfate were analyzed for the production of phytase and the results are shown in Figure 3b.

Among nitrogen sources, Ammonium sulfate, an inorganic nitrogen source, gave the best results (24.09±0.02 U/mL/min). Tian and Yuan (2016) reported identical results to our work from *Aspergillus ficuum* (12.93 ± 0.47 U/g ds). On the other hand, Singh (2017) contradicted with present findings and showed beef extract (47,432 U/L) as the best nitrogen source for immobilized phytase production from *Aspergillus oryzae* SBS50.

### *3.4.7 Inoculum size*

Five different inoculum sizes i.e. 1% to 5% were observed for their effect on phytase production and the results are shown in figure 3c. The inoculum size which showed the best results was 4%, giving 24.6±0.05 U/mL/min. Singh (2017) described similar results for *Aspergillus oryzae* SBS50 using 4% of inoculum. Similar inoculum sizes could be due to the nutrients present in medium taken up best by the cells at an inoculum size of 4%. On the other hand, Ajith *et al*. (2018) reported that optimum inoculum size for production was 5% for *Aspergillus foetidus* MTCC 11682.

## *3.5 Purification*

Precipitation of phytase occurred at an 80% fraction of ammonium sulfate. The enzyme activity and total protein in the supernatant were decreased (0.88±0.05 U/mL/min and 0 mg/ml) and in the pellet was increased (23.87±0.05 U/mL/min and 0.89±0.03 mg/mL), respectively are illustrated in figure 4(a). For further purification, the anion exchange chromatography was carried out. We obtained our desired peak between 47-50 min run time as illustrated in figure 4(b). The purified enzyme fraction then further analyzed for molecular weight determination using SDS-PAGE. The 53 kDa single band on the gel showed the molecular weight of phytase, as shown in figure 4(c).

The ammonium sulfate was used for the precipitation can partially purify the enzyme. The work of Tang *et al*. (2018) showed that *Aspergillus niger* N25 was precipitated at 80%.

Bekalu *et al*. (2017) reported that phytase was purified by obtaining clear supernatant at 60% by Aspergillus ficcum. After anion exchange chromatography, 1.93 folds increased the protein purification, and specific activity by 53.5 U/mg was recorded.

Saxena *et al*. (2019) suggested the use of DEAE Sepharose, and Sephadex G-100 size-exclusion column chromatography to purify phytase from Aspergillus aculeatus. Sanni *et al*. (2019) used DEAE Sephacel and Sephacryl S-200 anion exchange column from *Aspergillus fumigatus* and reported approximately 45%-fold purifications.

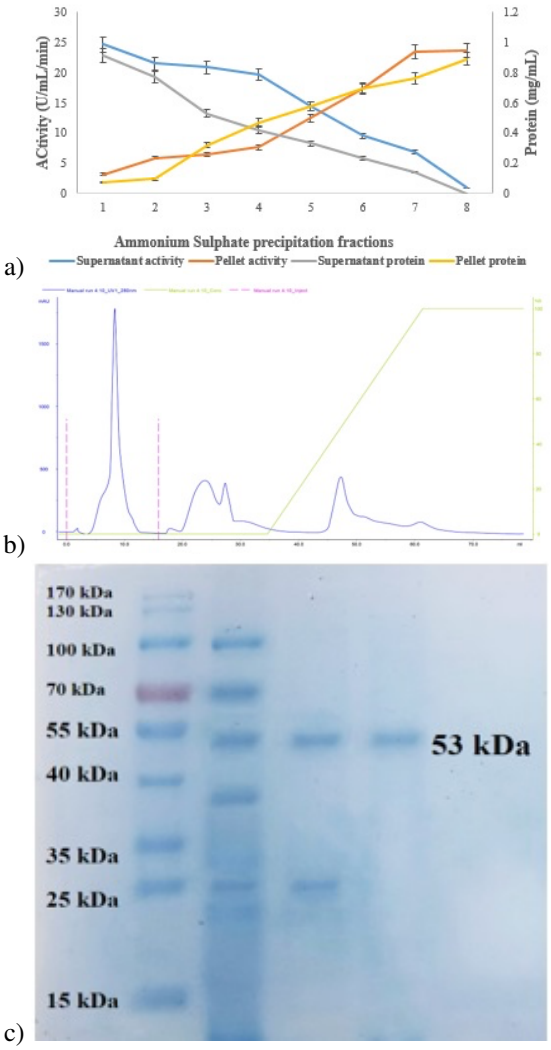



Fig. 4. (a) Ammonium sulphate precipitation of crude enzyme. Y-error bars represents the standard deviation (SD≤ ±0.05) between three replicates. (b) Purification of phytase by Hitrap QFF column using Bio-Rad UnosphereTMQ (BioscaleTM mini). (c) SDS-PAGE analysis of purified phytase (Lanes Left to Right; 1 (Ladder), 2 (Crude enzyme), 3 (After ammonium sulphate precipiataion), 4 (Purified fraction)).

Table 2. Purification summary of phytase.

| Sample | Total units (U) | Total proteins (mg) | Specific activity (U/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Crude enzyme | 24600 | 890 | 27.6 | 100 | 1 |
| Ammonium sulphate precipitation | 23870 | 685 | 34.8 | 97 | 1.26 |
| Anion exchange chromatography | 22100 | 413 | 53.5 | 89.8 | 1.93 |

Table 3. Thermodynamic profiling of phytase.

| Temperatures (ºC) | Thermodynamic parameters | | |
|---|---|---|---|
| | $E_a$ KJ/mol | $\Delta H$ KJ/mol | $\Delta S$ J/mol K |
| 25 | 15.73 | 13.12 | -169 |
| 27.5 | 16.01 | 13.98 | -169.75 |
| 30 | 16.84 | 14.32 | -170 |
| 32.5 | 16.37 | 14.02 | -170.12 |
| 35 | 15.99 | 13.71 | -170.34 |

The molecular weight of phytase was determined as 53 KDa by SDS- PAGE. Neira *et al*. (2020) reported that fungal phytase from *Aspergillus niger* has molecular weight 89 kDa. Deepika *et al*. (2018) reported that fungal phytase from *Aspergillus ficuum* MTCC 7591 has molecular weight 65 kDa. Sandhya *et al*. (2019) determined molecular weight from *A. niger* phytase S2 as 50 KDa. The current finding did not match with reported results because the molecular weight of protein depends upon the sources from which they were extracted. The overall purification profile of phytase purified is shown in table 2.

### *3.6 Substrate specificity and kinetic parameters*

Sodium phytate was considered as a highly specific substrate for phytase. Line weaver-Burk plot studied $K_m$ and $V_{max}$, as depicted in figure 5. Kinetic parameters i.e. $K_m$ and $V_{max}$ were determined as 3.41 mM and 24.6±0.05 U/mL/min, respectively. Neira *et al*. (2018) proposed that the phytase from *Aspergillus niger* showed broad substrate specificity, and reported $K_m$ value of 220 $\mu$M and a $V_{max}$ of 25 $\mu$M/min. Sanni *et al*. (2019) documented $K_m$ and $V_{max}$ for *Aspergillus fumigatus* (phytase) as 35.7 $\mu$mol/min and 7.2 mM, respectively. Since $K_m$ value is a depiction of the affinity of the enzyme for its substrate, so its lower values for phytase showed its higher affinity towards the substrate. $V_{max}$ showed a higher rate of the substrate to product conversion.

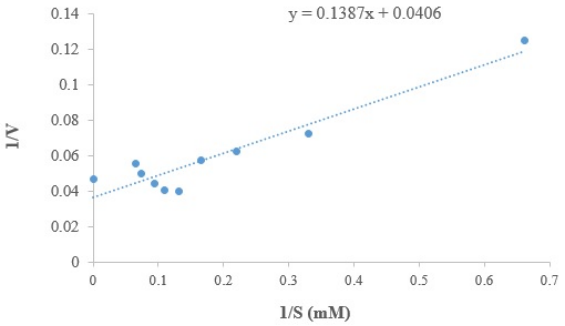
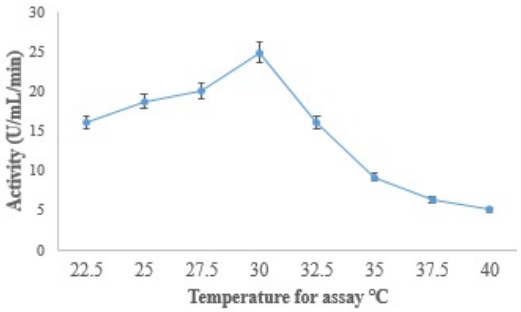


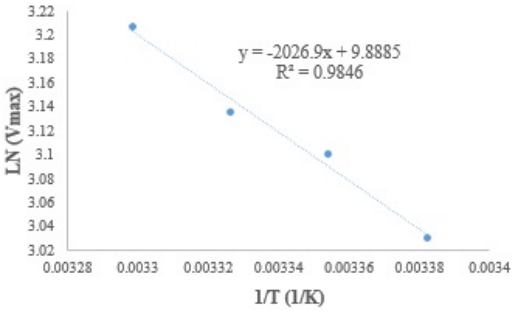

Fig. 5. Line weaver-Burke Plot to calculate the $K_m$ and $V_{max}$ for Phytase.

### *3.7 Effect of temperature on the activity*

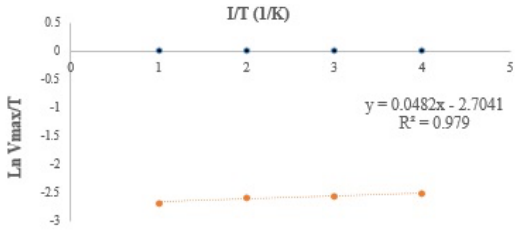
At different temperatures (22.5-40 ºC), enzyme activity was checked to observe its effect on enzyme activity. The maximum value (23.87±0.05 U/mL/min) was obtained at 30 ºC as shown in figure 6(a). The thermodynamic analysis was carried out and Ea (activation energy), ΔH (enthalpy of activation) and ΔS (change in entropy) were measured as 16.84 KJ/mol, 14.32 KJ/mol and -170 J/mol K, respectively, by Arrhenius plot, as shown in figure 6(b) and 6(c). Gnanwa *et al*. (2014) reported Ea, ΔH, and ΔS as 65.405 KJ/mol, 62.679 KJ/mol, and -83.31 J/mol K, respectively. Zhu *et al*. (2019) described the activation energy of phytate as 84.54 KJ/mol. The lower the values of thermodynamic analysis the more feasible




a)




b)




c)

Figure 6. (a) Temperature effect on Phytase activity. Y-error bars represents the standard deviation ($SD \leq \pm 0.05$) between three replicates. (b) Arrhenius plot to calculate the activation energy ($E_a$) and the enthalpy of activation ($\Delta H$) of Phytase. (c) Arrhenius plot to calculate the entropy of activation ($\Delta S$) of Phytase.

a product is for upscaling. The overall thermodynamic analysis is depicted in table 3.

### *3.8 Effect of temperature on the stability*

At different temperatures i.e. 30 ºC, 40 ºC, 50 ºC, and 60 ºC, the enzyme was incubated for 8 hours, and its stability was checked. At 0-time residual activity was 100%. At 30 ºC the residual activity was 98% for 5 hours and after 6 hours 80% of residual activity was maintained. While at 40 ºC enzyme had residual activity 85% for 2 hours after which it decreases up to 5 hours and then was maintained at 60% after 6 hours. At 50 ºC and 60 ºC enzyme was not suitably stable and a gradual decrease in residual activity was observed as shown in figure 7.

Thermostability is an important attribute to check whether enzymes can withstand inactivation at high

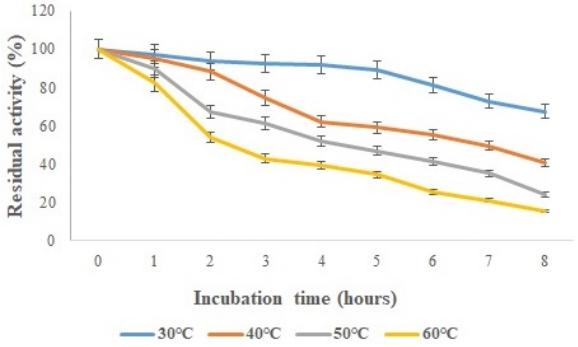
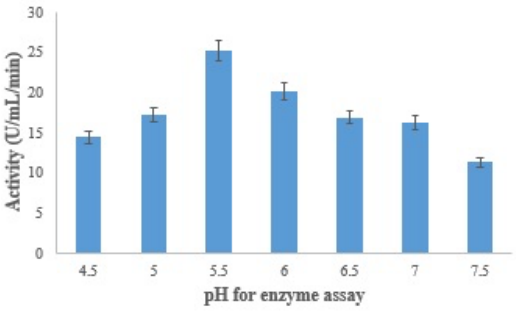
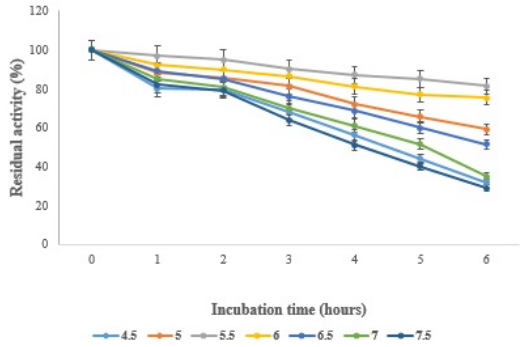



Figure 7. Effect of temperature on phytase stability. Y-error bars represents the standard deviation ($SD \leq \pm 0.05$) between three replicates.




a)




b)

Figure 8. (a) Effect of pH on phytase activity. (b) Effect of pH on phytase stability. Y-error bars represents the standard deviation ($SD \leq \pm 0.05$) between three replicates.

temperatures or not. Phytase produced from *A. oryzae* displayed optimum activity (23.87±0.05 U/mL/min) at 30 ºC and showed 98% stability for 5 hours. Buddhiwant *et al.* (2016) suggested that *A. niger* phytase was thermally stable. Zhou *et al.* (2019) also suggested that *A. neoniger* phytase was thermally stable. Ajith *et al.* (2019) proposed that *A. foetidus* showed more thermal stability than *A. niger* after immobilization.

### *3.9 pH effect on activity and stability*

The phytase activity was checked at different pH by using different ranges of pH (4.5-7.5) and the results are represented in figure 8(a). The stability of phytase enzyme activity was examined for 6 hours. At pH 5.5 and 6, the phytase was 90% stable for 6 hours. At pH 5 and 6.5, 85% residual activity was observed until 3 hours after that it started to decrease up to 60% until 6 hours. The enzyme was not significantly stable at pH 4.5, 7, 7.5 and residual activity considerably decreased up to 6 hours as illustrated in figure 8(b).

The optimal pH for the working of an enzyme was found out to be 5.5 (24.12±0.05 U/mL/min). At pH 5.5 and 6, phytase was 90% stable for 6 hours. Neira *et al*. (2018) reported closely related results using *Aspergillus niger*. Ajith *et al*. (2019) reported that phytase activity and stability from *Aspergillus foetidus* MTCC 11682 declined at high pH.

## Conclusions

It is concluded from the present study that phytase can be purified efficiently using the cascade of ammonium sulfate precipitation and anion exchange chromatography. The biosynthesis process optimization finding of the current study will help in developing an economical process in the future for industrial-scale biosynthesis of phytase, which can be utilized in different areas including poultry as a feed supplement thus having major significance for human ecologists and nutritionists. Considering the distinctive biological properties of fungal phytase, improving phytase activity and stability levels for dietary and industrial purposes is very much needed now. Furthermore, cloning and protein engineering of potential phytase producing fungal species will likewise be amazingly valuable for various industries.

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