PRODUCTION OF PHYTASE BY *ASPERGILLUS TERREUS* AND EVALUATING ITS STABILITY

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ABSTRACT: Secretory capacity of fungi, *Aspergillus terreus* (*A. terreus*), for phytase production is up to the mark. Soil samples containing *A. terreus* (n=20) were randomly collected in sterile plastic zipper bags, and recognized microscopically and morphologically to screen for phytase production. In the present study, *A. terreus* phytase production was quantified by providing different physical conditions *i.e.* temperature and pH to get their optimum level. Thermal stability was obtained by measuring stability at temperature range of 35°C, 55°C, 75°C, and 95°C, whereas, pH stability was estimated at intervals of 2h, 4h, 6h, and 8h. Chemical stability of phytase was judged by analyzing the effects of common earth crust metal ions such as Na^{+1}, K^{+1}, Ba^{+2}, Ca^{+2}, Cu^{+2}, Mg^{+2}, Mn^{+2}, and Fe^{+3}. Phytase production was assessed on phytase screening medium to observe the zone of hydrolysis. The highest phytase production was given by sample 16 (PAST-16) which was; 271.49 ± 8.14 units/mL (FTU/mL) and the lowest was given by sample 3 (PAST-3); 92.15 ± 5.53 FTU/mL. Temperature induced reduction in phytase enzyme and the highest reduction was noted at 95°C for 1 hour and the lowest reduction was recorded at 35°C for 15 min. Moreover, acidic pH (pH=2) induced more reduction for 1 hour exposure than high pH (pH=6) for 15 min. All metal ions, at their high concentration, adversely affected the phytase activity of *A. terrus* and Cu^{+2} showed the maximum reduction in phytase. Thus, the present study explored the physico-chemical stability of fungal phytase that can be used in poultry diet as a cheapest source of digestibility improving agent.

***Keywords*:** *Aspergillus* Phytase; Stability; Physical; Chemical analysis; In-vitro.

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INTRODUCTION

Phytic acid is an important part of plant-based food. Its inositol ring binding with 6 Phosphate (Pi) molecules. In this way, 60-90% Pi in legumes, seeds, and cereals is present in the form of Phytic acid (Sheikh *et al.,* 2012). It also acts as an anti-nutritional factor by chelating different metal ions, such as Ca, Mg, Zn, and Fe leading to mineral depletion. It creates a complex of proteins which reduce its digestibility and absorption in animal gut (Sheikh *et al.,* 2012). Most of the mono-gastric animals are deficient in Phytases so they are unable to completely hydrolyze the phytic acid to Pi and hence, consume it (Dailin *et al.,* 2018). The excretion of a large amount of Pi into soil and water causes eutrophication (Bekalu *et al.,* 2017).

Phytases are phosphatase enzymes that hydrolyze phytic acid into inositol and 6 Pi. So, it is widely used in animal feed to enhance the availability of Pi in feed (Correa and Araujo, 2020). Among microbial sources, fungal phytases are industrially important due to thermo-stability, pH tolerance, and resistance to proteases (Sheikh *et al.,* 2012). The thermo-stability of phytase is important because, during the feed pelleting process, the temperature may be reached temporarily from 60°C-90°C (Nascimento *et al.,* 2022). Moreover, phytases must be resistant to degradation by pH (2-8) in the digestive tract of poultry during the feed digestion process (Nascimento *et al.,* 2022).

The activity of phytases is influenced by metal ions that bind at different sites of enzymes and change structure of enzymes. Hence, these ions directly affect the enzyme activity (Liua *et al.,* 2021). The present study was conducted to evaluate the effects of different physicochemical factors on the stability and activity of phytases produced by *A. terreus* local isolates.

MATERIALS AND METHODS

Isolation and Identification of *A. terreus*: Soil samples (n=20) were randomly collected in sterile plastic zipper bags from the ground and plant nursery of University of veterinary and animal sciences, Lahore, Pakistan, in the month of July. From each soil sample, 200 mg was put off in 10 mL normal saline (0.9%) solution, placed in shaker at 150 rpm for 2 hours. 1 mL from the suspension

was then inoculated into sterile plates of Sabouraud's dextrose agar (SDA) and incubated for 5 days on 30 ℃. Isolates of *A. terreus* were identified by comparing macroscopic characteristics (diameter, overall front and reverse colors of colonies etc.).

Microscopic features of *A. terreus* isolates were observed by tease mount method of Gaddeyya *et al.*, (2012), cellophane tap method of Harris (2000) and slide culture method of Wijedasa and Liyanapathirana (2012) with minor modification to identify the genus. Types of hyphae and spores, color of hyphae and spores, presence of vesicles, metulea, phialids and presence of foot cell were observed under the microscope (MEIJI Techno).

Screening for Phytase Production: Phytate-screening medium (PSM) was composed according to method described by Qasim *et al*. (2017). It contain Glucose (1.5%), Sodium phytate (0.2%), Ammonium nitrate (0.2%), Potassium chloride (0.05%), Magnesium sulfate (0.05 %), Manganese sulfate (0.03%), Ferrous sulfate (0.001%) with pH 6.5-7. It was sterilized by autoclaving and Na-phytate was separately decontaminated by using Millipore 0.45µm membrane filter and added to autoclaved media aseptically.

PSM medium was inoculated with fungal spores and incubated at 30 °C, for 1 week (Qasim *et al.*, 2017). From this medium, fungal culture was procured and plated on PSM agar containing calcium phytate (0.5%) as phosphorous source. Incubation was given for 5 days at 30 °C. Isolates showed zone of hydrolysis on phytase screening agar were confirmed by double staining technique using method described by Mahmood *et al.* (2023). Cobalt chloride (2%) solution was flooded over *A. terreus* growth, incubated for 5 minutes, room temperature and removed. Solution of ammonium molybdate (6.25 %) and ammonium vanadate (0.42 %) 1:1 was poured over the culture, incubated for 5 minutes at room temperature and solution removed. Positive isolates were detected through presence of zone of hydrolysis around colony.

Toxigenic profile: The toxigenic profile of phytase-producing *A. terreus* isolates was determined by using method described by Yasmeen *et al.* (2021) with minor variation. Spores of fungi were inoculated in 100 mL SD broth, incubated in shaking incubator 25 ± 3°C) for 45 days. Mycotoxins were extracted and thin layer chromatography (TLC) was performed by using using TLC plates (20×20 cm) (Merck®) and mobile phase (chloroform: Acetone, 95: 5). Chromatograms were visualized under 365 nm wavelength.

Quantification of phytases: Phytase was produced from isolated positive for phytase production and negative for toxigenic screening, by submerging fermentation (Mahmood *et al.*, 2023). For this purpose 1 mL of standardized inoculum (10^6 spores/mL) was inoculated into PSM broth 50 mL and incubated at 28°C for 8-10 days under constant shaking (150 rpm). Phytases were extracted from PSM and quantified (Ajith *et al.*, 2019). Crude phytase (0.2 mL) was mixed with 0.2% sodium phytate (0.8 mL) prepared in sodium acetate buffer (0.2 M, pH 5.5) and incubated at 37°C for 30 minutes in the water bath. The reaction was stopped by adding 1 mL of tri-chloroacetic acid (5 %) solution. A colorimetric reagent (1 mL) was added to the mixture. Absorbance (optical density= OD) was measured at 655 nm and was correlated with a standard curve prepared from potassium di-hydrogen phosphate (KH_2PO_4) (Ajith *et al.*, 2019). One unit of phytase activity (FTU/mL) was defined as the amount of enzyme that released 1 µmol of phosphate per min under the assay conditions.

Stability profile of phytases: Thermo-stability of phytase was determined by incubating crude enzyme at temperatures 35°C, 55°C, 75°C and 95°C for 15, 30, 45, and 60 minutes (Ajith *et al.*, 2019) followed by phytase quantification, after exposure to each temperature for the required time. Tolerance of phytases to pH was assessed at pH 2.0, 4.0, 6.0, and 8.0 for 15, 30, 45, and 60 minutes, respectively. Quantification was carried out by phytase assay (Ajith *et al.*, 2019). The effect of metal ions on phytase was determined (Trivedi *et al.*, 2022). Concisely, crude enzyme and metal ions including Ba^{2+} ($BaCl_2$), Ca^{2+} ($CaCl_2$), Cu^{2+} ($CuSO_4$), Fe^{3+} ($FeSO_4.7H_2O$), K^+ (KCl), Mg^{2+} ($MgSO_4.7H_2O$), Mn^{2+} ($MnSO_4.7H_2O$) and Na^+ (NaCl) were mixed separately in equal volume (200µL). These mixtures were incubated at 4°C for 2 hours followed by phytase quantification (Ajith *et al.*, 2019). Three concentrations of metal ions (1, 5, and 10 mM) were evaluated for their effects on phytase activity.

Statistical analysis: All data was analyzed statistically by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range (DMR), posthoc test using statistical package for social sciences (SPSS) version 20.0.

RESULTS

Isolation and screening of phytase-producing *A. terreus:* The front view of *A. terreus* on SDA was initially white and turned to cinnamon brown in the center with a white periphery and cottony texture. The reverse view of the plate was yellow to tan in color. Microscopy showed septate hyaline hyphae and phialo-spores arranged in chains covering the vesicle (Fig. 1a, 1b).

All the 20 samples of *A. terreus* were found phytase producers (Fig. 1c). Zone of hydrolysis to colony ratio of PAST is presented in the Fig 2. The highest colony diameter and zone of hydrolysis were 36.00 ± 1.32 mm and 67.00 ± 2.29 mm for PAST-05 and PAST-01, respectively. The lowest colony diameter, due to zone

of hydrolysis, recorded were 13.00 ± 0.87 mm and 34.50 ± 0.50 mm for PAST-18 and PAST-07, respectively. The zone of hydrolysis to colony diameter ratio was 4.25 ± 0.34 mm which is maximum for PAST-02 and 1.70 ± 0.10 mm minimum for PAST-05, as shown in the Fig 2.

Toxigenic profile: Six isolates of *A. terreus*; PAST-03, PAST-05, PAST-06, PAST-09, PAST-15, and PAST-16 were detected as non-toxigenic and selected for further experiments (Fig. 1d).

Quantification of phytases: The *A. terreus* (PAST-16) showed maximum phytase activity (271.49 ± 8.14 FTU/mL) followed by PAST-15 (203.75 ± 4.47 FTU/mL), PAST-09 (117.98 ± 17.97 FTU/mL), PAST-06 (107.75 ± 10.23 FTU/mL), PAST-05 (79.00 ± 8.05 FTU/mL) and PAST-03 (92.15 ± 5.53 FTU/mL) (Fig. 3).

Stability profile of phytases: The stability profile in terms of phytase activity was determined by different physicochemical factors. The highest reduction (259.25 ± 0.84 FTU/mL) in phytase activity was found at 95°C for 60 min exposure. The lowest reduction (50.20 ± 7.36 FTU/mL) was recorded at 35°C for 15 min of exposure. Phytase lost stability with an increase in temperature and the exposure time to that temperature (Fig. 4).

Maximum reduction (206.14 ± 6.37) in phytase activity was observed at pH 2 for 60 min. The phytases remained more stable when exposed to pH 6 for 15 minutes as the lowest reduction (68.22 ± 10.37 FTU/mL) was observed at this pH (Fig. 5).

Metal ions; Na^{+}, K^{+}, Mn^{+2}, Ca^{+2} and Cu^{+2} showed dose dependent response. Reduction in FTU/mL was highest at a high concentration (10 mM) and lowest at (1 mM). However, the Cu^{2+} proved to be a strong inhibitor and reduced phytase activity up to 231.48 ± 3.68 at 10 mM concentration. The lowest reduction (45.32 ± 28.54) was shown by 1 mM K^{+} (Fig. 6).

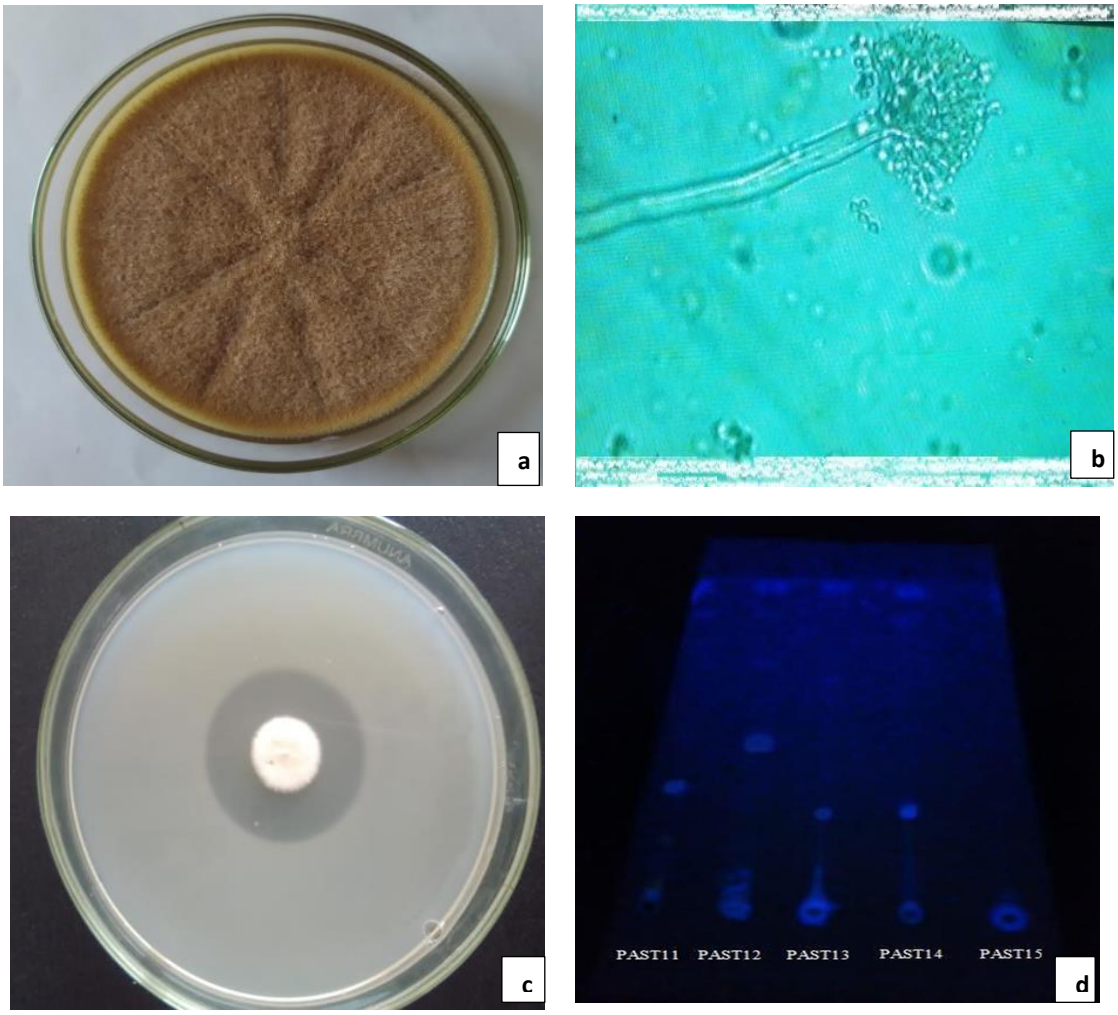

Figure 1:Isolation and identification of Phytase producing *Aspergillus terreus* from soil, a: Colony morphology (front view), b: Microscopic view at 400X, c: zone of hydrolysis on phytase screening medium, d: Toxigneic profile of isolates on TLC plate

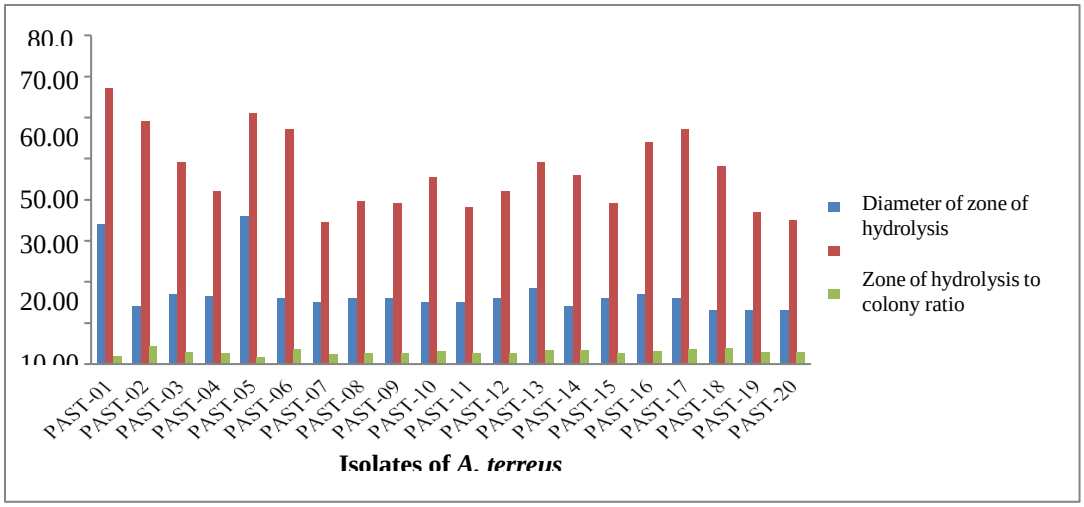

Figure 2: Screening phytase production in *Aspergillus terreus* isolates from soil. Chart showing colony diameters, phytic acid hydrolysis zones and zone to colony ratios of all 20 isolates (PAST1-PAST20).

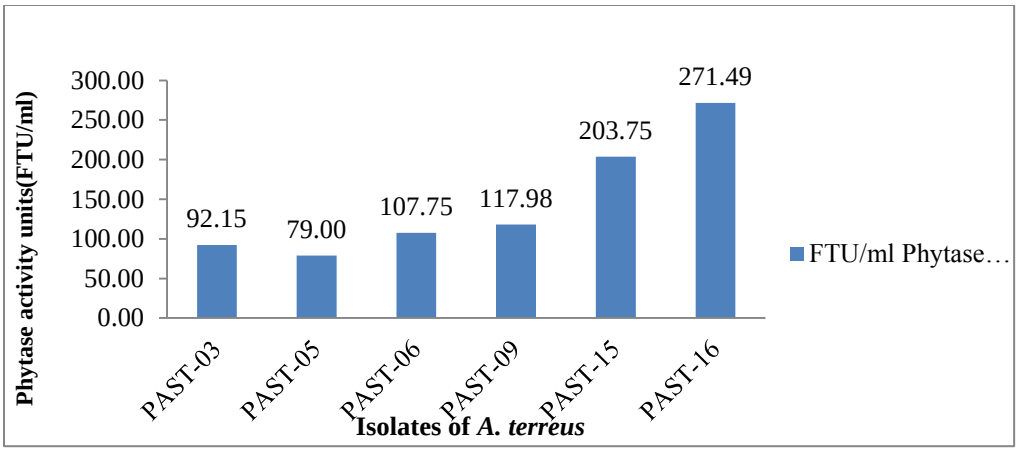

Figure 3: Quantitative analysis of phytase production potential of *Aspergillus terreus* isolate from soil (non-toxigenic).

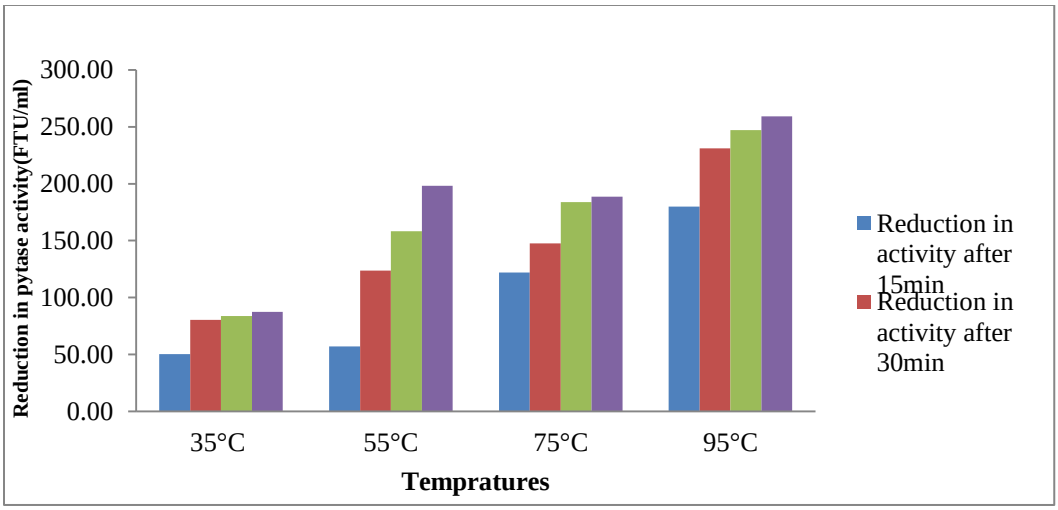

Figure 4: Effect of temperature on phytase activity (FTU/Ml) with relation to time. Phytase produced by isolates of *Aspergillus terreus* from soil.

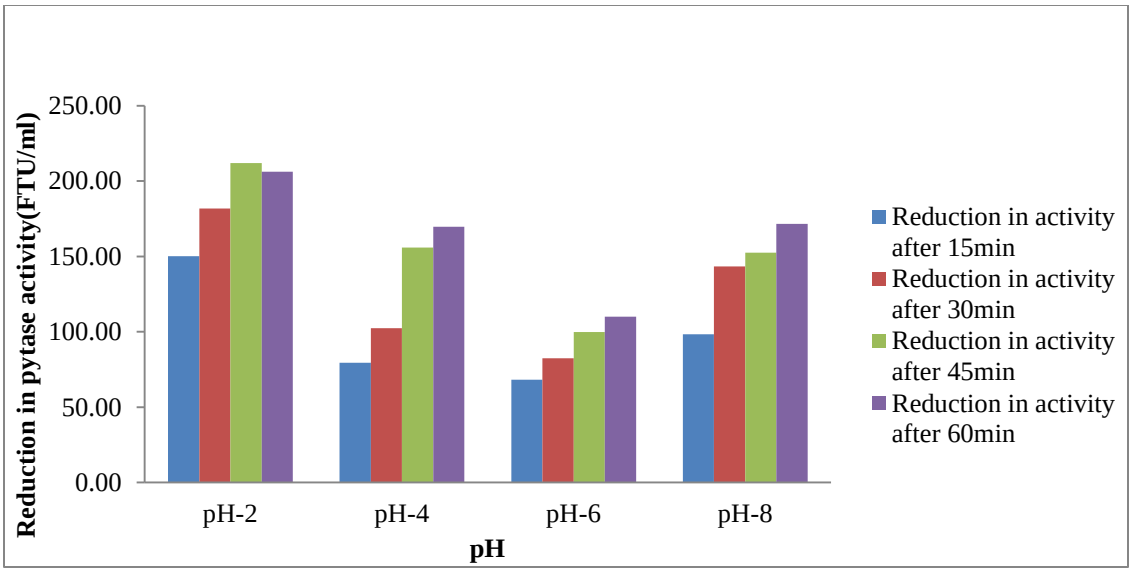

Figure 5: Effect of pH on phytase activity (FTU/Ml) with relation to time. Phytase produced by isolates of *Aspergillus terreus* from soil.

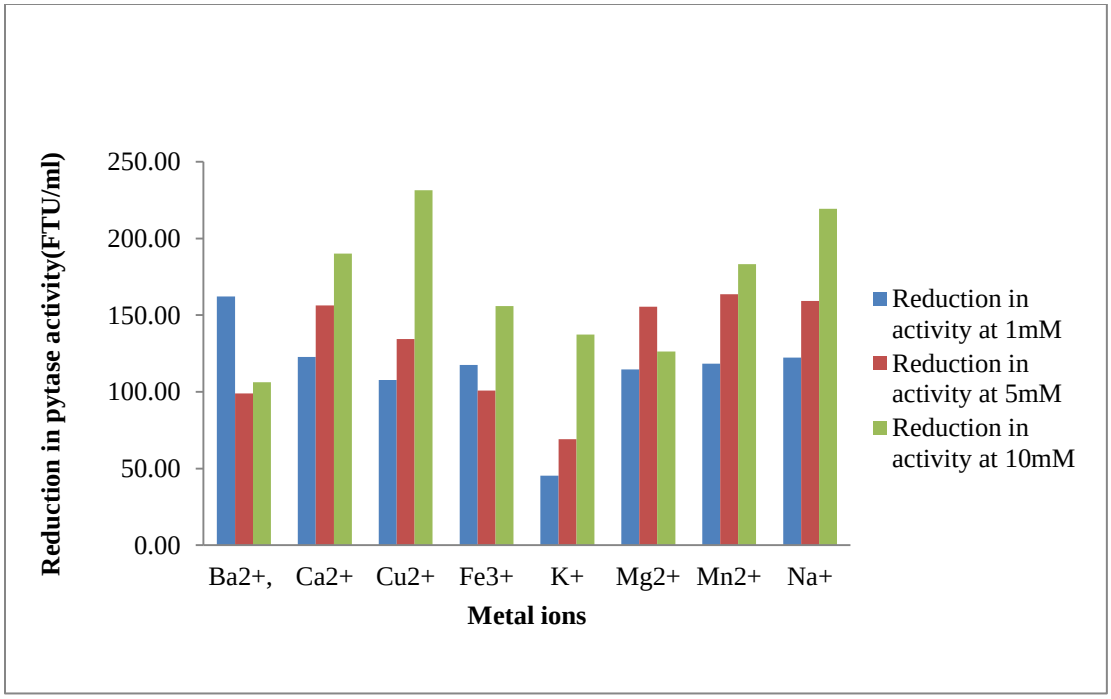

Figure 6: Reduction in phytase activity at different concentration of metal ions.

DISCUSSION

Indigenous filamentous fungi from the soil are capable of producing several industrially important extracellular enzymes. Different researchers reported phytase production by different *Aspergilli* including *A. niger* (Nascimento *et al.*, 2022), *A. flavus A. fumigatus* (Mishra *et al.*, 2013), *A. ficuum* (Bekalu *et al.*, 2017) and *A. terreus* (Mishra *et al.*, 2013). In the present study, *A. terreus* produced zones of hydrolysis from 67.00 ± 2.29 to 34.50 ± 0.50 mm on a phytase screening medium. Stability to physio-chemical factors in the digestive tract of animals is an important criterion for the industrial application of phytases (Monteiro *et al.*, 2015). The phytases of *A. terreus* were evaluated for *in vitro* stability to physio-chemical parameters due to the enhanced industrial influence of stable enzymes.

Several studies have been conducted on the thermos-stability of phytases produced from *Aspergillus sp*. The phytase from *Aspergillus sp.* was stable at a wide

range of temperatures (20°C-60°C) (Sadaf *et al.,* 2022). *A. niger UFV-1* retained 80% active at 60ºC for 120 hours (Monteiro *et al.,* 2015). The hytase from *A. ficuum* stable at 60°C with no loss in activity on exposure at 70°C for 20 min. However, activity was lost at 80°C after 10 min exposure (Zhang *et al.,* 2015). *F. verticillioides* producing phytases at 30ºC to 60ºC, however, 30% activity of phytases was lost at 60ºC. In the present study, it was a 66.23% reduction in the activity of phytase on exposure to 95ºC for 15 minutes (Marlida *et al.,* 2010).

It was revealed by recent research that Phytase from *Aspergillus sp.* was stable at a pH range of 4-8 (Sadaf *et al.,* 2022). The phytase produced by *A. niger* showed 50% enzyme activity in a wide range of pH (4-7) (Vielma *et al.,* 2018). In another study, 50%-100% activity of phytase produced by *F. verticillioides* was observed at pH 2.5-6.0 (Marlida *et al.,* 2010). The results of the above-mentioned studies are in agreement with the present where the minimum loss of phytase activity of *A. terreus* was observed at pH 6.0.

Phytase from *Aspergillus sp.* was insensitive to Ca^{2+}, Mg^{2+}, Ni^{2+}, and Ba^{2+}(Sadaf *et al.,* 2022). Phytase from *A. niger* showed 25%-30% activity in the presence of Mg2+, Mn2+, Cu2+, Cd2+, and Ba2+ (Vielma *et al.,* 2018). In another study, *A. niger UFV*-1 phytase was slightly inhibited by Mg^{2+}, Cd^{2+}, K^{+}, and Ca^{2+} at 10Mm concentration (Monteiro *et al.,* 2015). *A. niger* phytase NCIM 563 was inhibited in the presence of 1 mM Hg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, and Ca^{2+} (Bhavsar *et al.,* 2013). Phytases produced by *Penicillium simplicissimum* was inhibited by Fe^{2+}, Fe^{3+}, and Zn^{2+}. The results of the above-mentioned studies strengthen the present study findings in which the inhibitory effect of metal ions was observed on *A. terreus* phytases activity**.**

Conclusion: It was concluded that *A. terreus* could be a better source of stable phytase that can tolerate high temperatures during the pelleting process of poultry feed. It can also tolerate digestive tract pH. Its activity was inhibited by high concentrations of metal ions. This stable phytase can also be used in poultry feed at the commercial level to enhance the feed conversion ratio in poultry birds.

Author Contributions: Conceptualization, methodology and validation by S.K and A.A.A. Writing- original draft and editing By S.K and R.Y. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest

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