

# SCREENING AND CHARACTERIZATION OF POTENTIAL FUNGI FROM ENVIRONMENTAL SAMPLES FOR EXTRACELLULAR PHYTASE PRODUCTION

## ABSTRACT

Phytases are enzymes of significant interest due to their ability to hydrolyze phytic acid, thereby releasing inorganic phosphate and making it available for various biological processes. This study aimed at screening and characterization of potential phytase-producing fungi isolated from environmental samples for extracellular phytase production. For this study, various types of environmental samples were collected from various locations in the Khulna region. A total of 15 fungal isolates were isolated, and then morphological and microscopic characterization aided in the preliminary identification of fungal species. Screening for extracellular phytase production using a plate assay containing phytin as the sole phosphorus source was conducted. Five fungal isolates (Isolate 2, Isolate 3, Isolate 5, Isolate 11, and Isolate 12) provide positive results in the phytase screening test. Total protein concentration of these five fungal isolates was determined using TCA protein precipitation technique. Protein from Isolate 2 (8.84 µg/ml) and Isolate 12 (49.77 µg/ml) were selected by considering their higher protein concentration for the thermostability test. Isolate 2 and Isolate 12 both exhibit positive results with thermostable properties, which were tested by the gradual increased temperature tolerance rate (4°C-50°C) Phytase enzyme from Isolate 12 showed 40% higher temperature stability than that of isolate 2 Further studies aimed at the molecular characterization of isolate 12 and its use as feed additives. This research contributes to the discovery of potential fungal phytases with enhanced industrial potential in various applications.

*Keywords: Screening, Characterization, Potential, Phytase, Extracellular enzyme*

## 1. INTRODUCTION

Phytic acid, also known as myo-inositol 1,2,3,4,5,6-hexakisphosphate, is a primary storage form of phosphate found in grains, legumes, and oilseeds, comprising 60–80% of total phosphorus (P). These crops, particularly cereal grains and oilseed meals, form the core of animal feed. If monogastric animals like pigs and poultry could efficiently utilize the phosphorus from phytate, the phosphorus content in these feeds would be sufficient for optimal growth [1]. However, monogastric animals lack the necessary enzymes to break down phytate, leading to the excretion of phytate-bound phosphorus in their manure, resulting in both nutrient deficiencies and environmental pollution [2]. Phytase enzymes (EC 3.1.3.8 and EC 3.1.3.26) play a critical role in overcoming this challenge. These enzymes hydrolyze phytic acid and its salts, releasing inositol, inositol monophosphate, and inorganic phosphate [3]. As a result, phytases are added to

animal feed to enhance the absorption of phosphorus and minerals [15], while also reducing the amount of phosphate excreted [2]. Phytases are found across various organisms, including plants, animals, bacteria, yeasts, and filamentous fungi, with microbial phytase, particularly from fungi, being the primary enzyme used in animal feed due to its efficiency [4].

Fungal phytases, especially those produced by species such as *Aspergillus niger*, *Mucor piriformis*, and *Cladosporium*, have become a major focus for industrial production due to their extracellular nature and higher thermostability compared to bacterial phytases [5, 6]. These fungi are considered ideal for large-scale phytase production, with over 200 fungal isolates showing active extracellular phytase production [7].

Thermophilic fungi, in particular, have shown promise in producing thermostable phytase, which is essential for industrial processes that require enzymes to function under extreme conditions [8]. Fungal phytases offer advantages such as greater thermal stability and activity in acidic environments, making them suitable for use in animal feed [2]. Furthermore, genetic engineering has enabled the development of phytases with enhanced properties [23, 25], such as improved temperature stability and pH tolerance, further increasing their effectiveness in food and feed industries [6, 24].

The demand for phytase enzymes continues to grow due to their broad applications, including improving nutrient bioavailability in human food, enhancing animal nutrition, and mitigating phosphorus pollution [9]. With ongoing research and technological advancements, the potential for developing more efficient and stable phytases for industrial use remains a promising field of exploration [4].

## 2. material and methods

### 2.1 Sample Preparation and Isolation of Fungal Strains

Soil samples (1 g) were suspended in 9 mL of distilled water and vortexed thoroughly. Serial dilutions ranging from  $10^{-1}$  to  $10^{-5}$  were prepared, and aliquots of 100  $\mu$ L, 200  $\mu$ L, 300  $\mu$ L, 400  $\mu$ L, and 500  $\mu$ L were plated onto sterilized Potato Dextrose Agar (PDA), which had been autoclaved at 15 psi, 121°C for 20 minutes. Plates were incubated at 30°C for 3 days at 150 rpm. The same procedure was applied to washed soil samples. After 3 to 4 days, distinct fungal cultures were isolated [12]. Individual fungal spores were carefully picked using a flame-sterilized needle and re-inoculated onto fresh PDA plates. The plates were incubated again at 30°C for 3 days to establish pure fungal cultures. In total, 15 fungal species were isolated from the environmental samples and maintained for further study [11].

### 2.2 Morphological and Microscopic Identification of Fungal Strains

The preliminary identification of the fungal strains was carried out through morphological characterization, which included observations of colony color, texture, and growth patterns [18]. Further microscopic analysis was performed to examine key fungal structures. Hyphae characteristics such as the presence or absence of septa, branching patterns, and hyphal width were noted. Reproductive structures, including conidiophores and conidia, were observed for their size, shape, and arrangement. These observations provided valuable clues for the potential identification of the fungi and helped focus subsequent investigations [19].

To screen for phytase production, the fungal strains were inoculated onto PDA plates containing 2% phytin as the sole phosphorus source. The plates were incubated at 30°C, and the zone of inhibition was measured at 24-hour intervals over a 96-hour period. From this screening process, five fungal species demonstrated significant phytase

activity by producing clear zones of inhibition around the fungal colonies [20].

### **2.3 Extracellular Fungal Protein Precipitation**

Extracellular proteins were precipitated from fungal cultures that had been grown for two days in a shaking incubator. The cultures were centrifuged at 3000 rpm to pellet the cells, and the supernatant containing the extracellular proteins was collected [21]. A 10% trichloroacetic acid (TCA) solution was added to the supernatant, and the mixture was incubated on ice for 1 hour to precipitate the proteins [22]. After centrifugation at 10,000 rpm, the protein pellet was washed with ice-cold acetone to remove any residual TCA and debris. The pellet was then resuspended in 100  $\mu$ L of PBS buffer [16].

### **2.4 Determination of Fungal Protein Concentration**

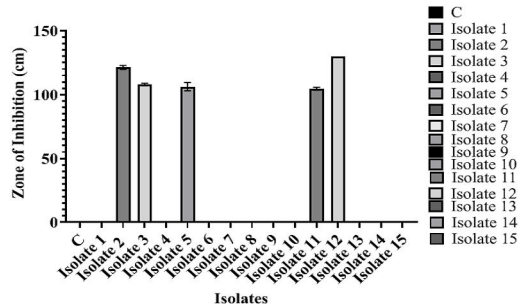
The fungal protein concentration was determined using a NanoDrop spectrophotometer at the Acharya Prafulla Chandra Ray Central Laboratory (APCRCL) of Khulna University, Bangladesh. The NanoDrop provided rapid and accurate measurements of protein concentration from a small 2  $\mu$ L sample, allowing for efficient protein quantification [13].

### **2.5 Thermostability Testing of Fungal Proteins**

A thermostability test was conducted to assess the heat resistance of the fungal proteins. Two approaches were used: temperature-treated and non-temperature-treated samples. In the temperature-treated method, 700  $\mu$ L of the fungal protein was incubated at room temperature (25°C) for 30 minutes, followed by inoculation onto PDA plates and incubation at 30°C for 18 hours. The process was repeated for different temperatures, including 40°C and 50°C, and the zone of inhibition was measured to evaluate the effect of heat on protein stability. For the non-temperature-treated samples, 700  $\mu$ L of fungal protein was directly inoculated onto PDA plates and incubated at 25°C, 40°C, and 50°C for 18 hours. The zones of inhibition observed in both treated and untreated samples were used to determine the thermostability of the fungal proteins across different temperature conditions.

## **3. results and discussion**

This research was conducted to screen and characterize potential fungi from environmental samples for extracellular phytase production. Environmental samples were collected from the Khulna district, near the Khulna University area. A total of 15 fungal isolates were obtained from these samples, and pure cultures were established for further study. Morphological and microscopic characterizations of the 15 isolates are shown in Fig1. These isolates came from various sources, including soil, plants, fruits, and other organic matter.



**Fig. 1. Phytase Producing Fungal Isolates**

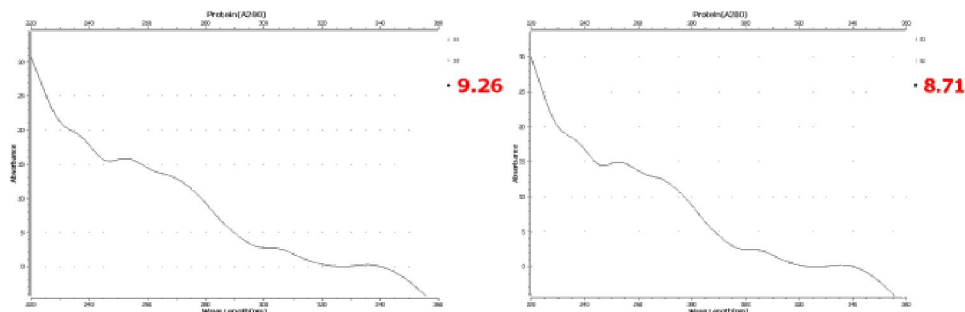
Among the 15 fungal isolates, five showed positive results in the phytase screening test by producing a significant zone of inhibition in the phytin-enriched selective media. The solubilization index (SI) and solubilization efficiency (SE) of these five isolates were calculated using the formula:

$$SI = \frac{\text{Total diameter} - \text{Colony diameter}}{\text{Total diameter}}$$

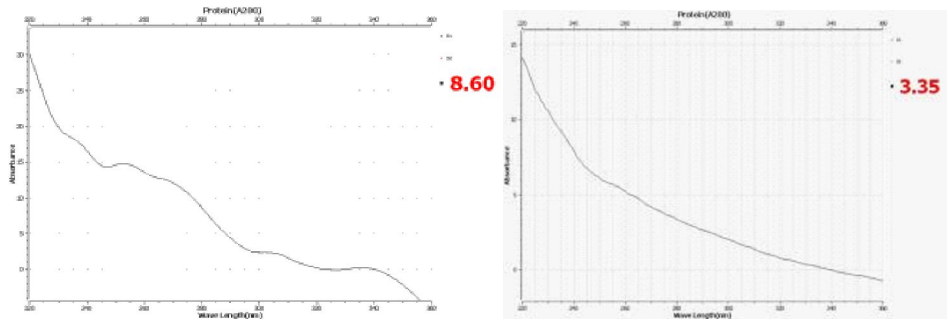
$$SE = \frac{\text{Total diameter} - \text{Colony diameter}}{\text{Total diameter}} \times 100$$

The data collected from these calculations indicated the efficiency of phytase production by these isolates, as shown in Fig1.

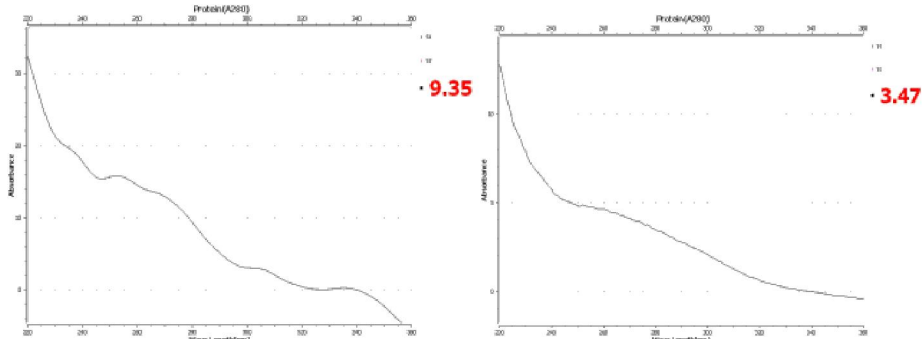
The protein concentration of the five phytase-producing isolates was determined through extracellular fungal protein precipitation. The concentrations were measured using a NanoDrop spectrophotometer, and results from two replicas were recorded. Isolate 2 showed protein concentrations of 9.26 µg/mL and 8.71 µg/mL, Isolate 3 had 8.60 µg/mL and 3.35 µg/mL, Isolate 5 showed 9.35 µg/mL and 3.47 µg/mL, Isolate 10 had 8.70 µg/mL and 4.61 µg/mL, and Isolate 11 had 9.66 µg/mL and 9.88 µg/mL. Among these, Isolate 2 and Isolate 12 exhibited the highest protein concentrations (Fig.2. – Fig.7.). Further analysis revealed that Isolate 2 had protein concentrations of 9.79 µg/mL, 8.00 µg/mL, and 8.71 µg/mL, while Isolate 12 exhibited even higher concentrations at 54.72 µg/mL, 39.89 µg/mL, and 54.7 µg/mL, as shown in Fig.8.– Fig.10.



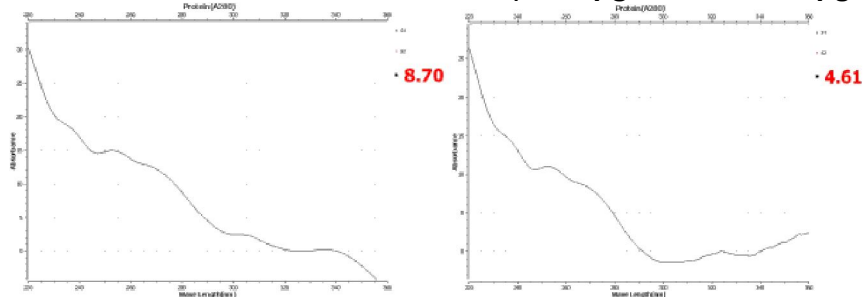
**Fig.2. Protein concentration of isolate 2 (9.26 µg/mL and 8.71 µg/mL).**



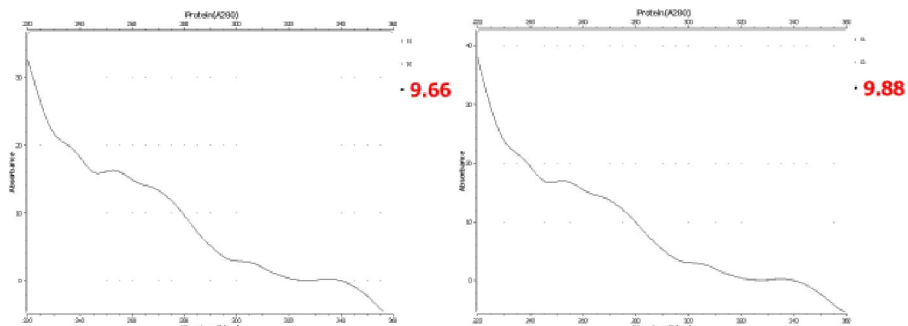
**Fig.3. Protein concentration of isolate 3 (8.60 µg/mL and 3.35 µg/mL).**



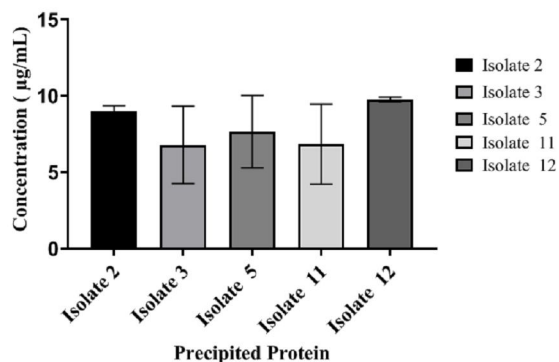
**Fig.4. Protein concentration of isolate 5 ( 9.35 µg/mL and 3.47 µg/mL ).**



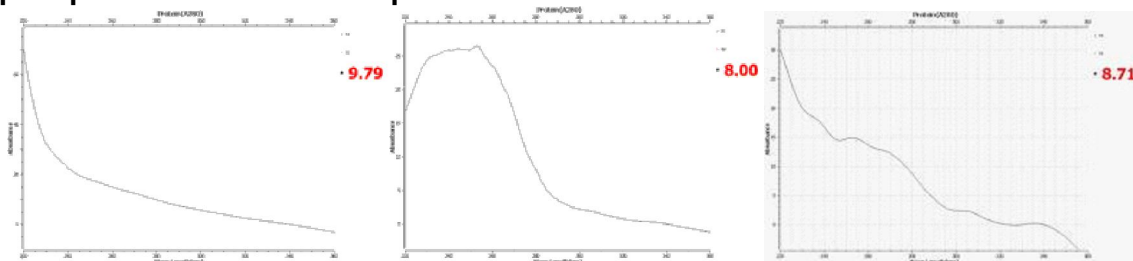
**Fig.5. Protein concentration of isolate 11 (8.70 µg/mL and 4.61 µg/mL).**



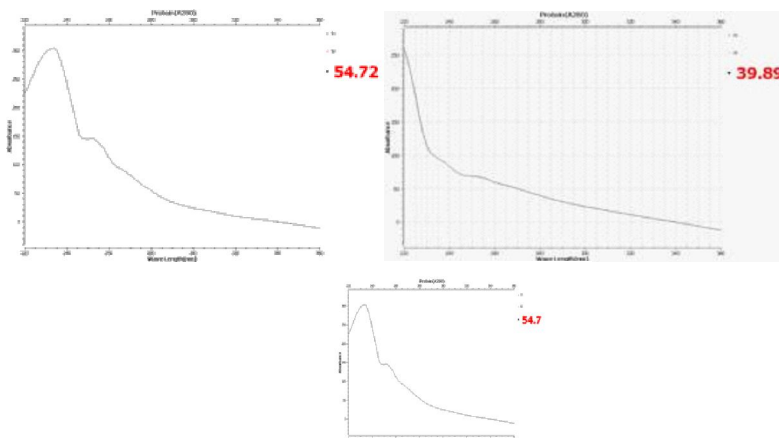
**Fig.6. Protein concentration of isolate 12 (9.66 µg/mL and 9.88 µg/mL).**



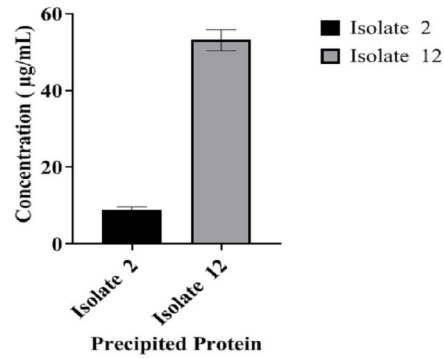
**Fig.7. Total protein concentration of 5 different phytase-producing isolates. Among these five isolates, isolate 2 and isolate 12 showed a high protein concentration. Data were obtained from the extracellular fungal protein precipitation method and expressed as mean  $\pm$  SD.**



**Fig.8. Protein concentration of isolate 2 (9.79 µg/mL, 8.00 µg/mL and 8.71 9.66 µg/mL).**



**Fig.9. Protein concentration of isolate 12 (54.72 µg/mL, 39.89 µg/mL, and 54.7 µg/mL).**

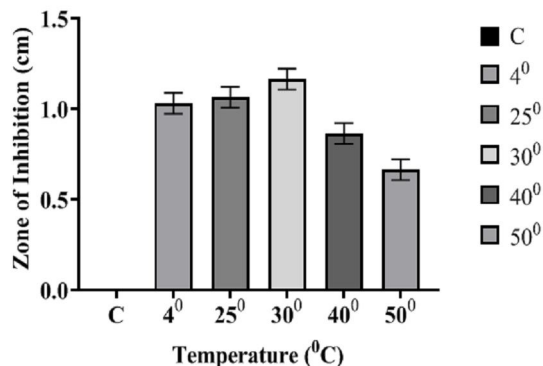


**Fig.10. Total protein concentration of 2 different phytase-producing isolates. Between these two isolates, isolate 12 showed a high protein concentration. Data were obtained from the extracellular fungal protein precipitation method and expressed as mean  $\pm$  SD.**

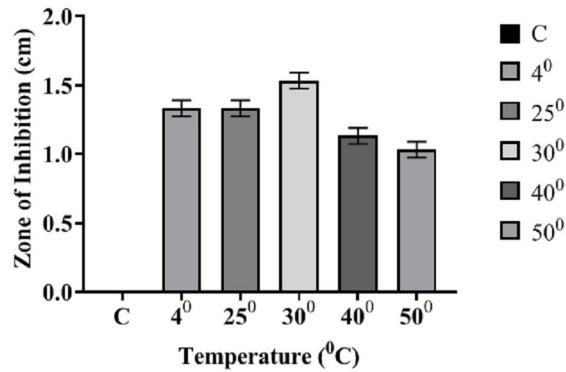
Thermostability tests were conducted to evaluate the heat resistance of the phytase produced by Isolates 2 and 12. These isolates were subjected to temperature treatments at 40°C, 25°C, 30°C, 40°C, and 50°C for 30 minutes, followed by inoculation into phytin-enriched media. The highest zone of inhibition for both isolates was observed at 30°C, with Isolate 12 showing a greater inhibition zone than Isolate 2 (Fig.11. – Fig.13.).



**Fig.11. Thermostability test (temperature treatment) of isolate 2 and isolate 12 at 30°C temperature.**



**Fig.12. Temperature-dependent treatment of the thermostability test of isolate 2, conducted at 4° C, 25° C, 30° C, 40° C, and 50° C, and the highest zone of inhibition appeared at 30° C. Data were obtained from plate assay method and expressed as mean  $\pm$  SD.**

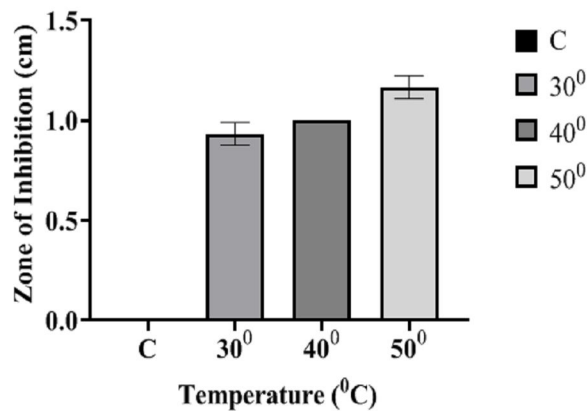


**Fig.13. Temperature-dependent treatment of the thermostability test of isolate 12, conducted at 4° C, 25° C, 30° C, 40° C, and 50° C, and the highest zone of inhibition appeared at 30° C. Data were obtained from the plate assay method and expressed as mean ± SD.**

When the isolates were tested without temperature treatment, direct inoculation of protein at 300°C, 400°C, and 500°C was conducted. In this case, the highest zone of inhibition was observed at 50°C for both isolates, with Isolate 12 again showing a larger inhibition zone than Isolate 2 (Fig.14– Fig.16.). A comparison of the two isolates under both temperature-treated and untreated conditions is shown in Fig.17.

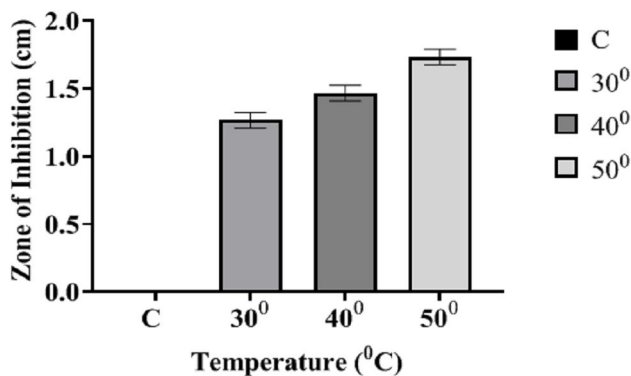


**Fig.14: Thermostability test (direct inoculation) of isolate 2 and isolate 12 at 50°C temperature.**

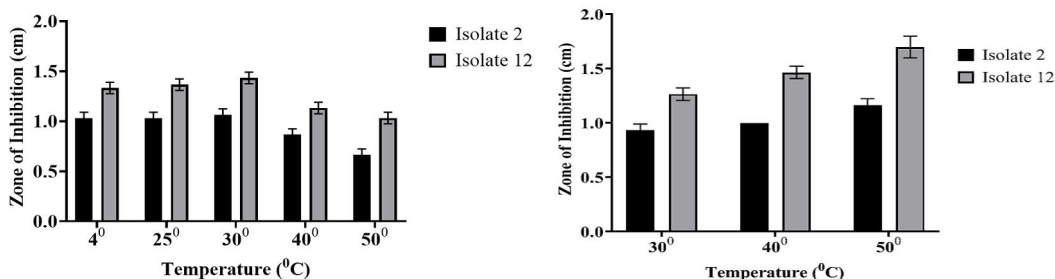


**Fig.15. Thermostability test of isolate 2 (without temperature treatment),**

conducted at 30<sup>o</sup> C, 40<sup>o</sup> C, and 50<sup>o</sup> C, and the highest zone of inhibition appeared at 50<sup>o</sup> C. Data were obtained from the plate assay method and expressed as mean ± SD.



**Fig.16.** Thermostability test of isolate 12 (without temperature treatment), was conducted at 30<sup>o</sup> C, 40<sup>o</sup> C, and 50<sup>o</sup> C, and the highest zone of inhibition appeared at 50<sup>o</sup> C. Data were obtained from the plate assay method and expressed as mean ± SD.



**Fig.17.** Comparison between Isolate 2 and Isolate 12 at temperature treatment and without temperature treatment. In these two tests, Isolate 2 showed a higher value in the zone of inhibition than isolate 2.

The results of this study demonstrate the potential of these fungal isolates for phytase production. Both Isolate 2 and Isolate 12 were capable of producing significant zones of inhibition in the phytin-enriched media under various conditions, indicating their thermostability. Isolate 12, in particular, showed higher protein concentrations and greater inhibition zones than Isolate 2 under both temperature-treated and untreated conditions.

The study highlights the successful isolation of phytase-producing fungi from diverse environmental samples, supporting previous findings that fungi capable of producing phytase are readily found in soils, plant material, and organic waste [2]. Isolate 12 stood out in terms of both protein concentration and thermostability, demonstrating its potential for industrial applications, especially in processes requiring heat-resistant enzymes. Both Isolates 2 and 12 demonstrated stability at elevated temperatures (up to 50°C), which is consistent with the findings from [10] regarding fungal phytases with thermostable properties [14].

#### 4. Conclusion

The screening and characterization of thermostable fungi for extracellular phytase production represent a significant advancement in biotechnological research, with promising implications for sustainable agriculture and environmental management. These findings contribute to the broader understanding of fungal enzymology and offer potential solutions to challenges related to nutrient availability and environmental sustainability. This study explored the potential of environmental samples (soil, fruits, vegetables) as sources for thermostable fungal species capable of producing extracellular phytase. Among the fifteen isolates, Isolate 2 and Isolate 12 demonstrated notable thermostable properties across various temperature ranges, with Isolate 12 showing superior stability. These results pave the way for further studies focused on the biochemical and molecular characterization of Isolate 12, a crucial preliminary step towards its potential application as a feed additive and its production on an industrial scale.

#### References

- Gontia-Mishra I, Deshmukh D, Tripathi N, Bardiya-Bhurat K, Tantwai K, Tiwari S. Isolation, morphological and molecular characterization of phytate-hydrolysing fungi by 18S rDNA sequence analysis. *Brazilian Journal of Microbiology*. 2013;44(1):317-23. DOI: 10.1590/S1517-83822013000100047.
- Ocampo Betancur M, Patiño Cervantes LF, Marín Montoya M, Salazar Yepes M, Gutiérrez Sánchez PA. Isolation and characterization of potential phytase-producing fungi from environmental samples of Antioquia (Colombia). *Revista Facultad Nacional de Agronomía Medellín*. 2012;65(1):6291-303.
- Casey A, Walsh G. Identification and characterization of a phytase of potential commercial interest. *Journal of Biotechnology*. 2004;110(3):313-22. DOI: 10.1016/j.jbiotec.2004.02.017.
- Bhavsar K, Khire JM. Current research and future perspectives of phytase bioprocessing. *RSC Advances*. 2014;4(51):26677-91. DOI: 10.1039/C4RA02430A.
- Singh B, Satyanarayana T. Fungal phytases: characteristics and amelioration of nutritional quality and growth of non-ruminants. *Journal of Animal Physiology and Animal Nutrition*. 2015;99(4):646-60. DOI: 10.1111/jpn.12246.
- Singh N, Kuhar S, Priya K, Jaryal R, Yadav R. Phytase: The feed enzyme, an overview. In: Yadav S, editor. *Advances in Animal Biotechnology and its Applications*. New Delhi: Springer; 2018. p. 269-327.
- Saroj P, Narasimhulu K. Characterization of thermophilic fungi producing extracellular lignocellulolytic enzymes for lignocellulosic hydrolysis under solid-state fermentation. *Bioresources and Bioprocessing*. 2018;5(1):1-14.
- Dailin DJ, Hanapi SZ, Elsayed EA, Sukmawati D, Azelee NIW, Eyahmalay J, et al. Fungal phytases: biotechnological applications in food and feed industries. In: El Enshasy H, editor. *Recent Advancement in White Biotechnology Through Fungi: Volume 2: Perspective for Value-Added Products and Environments*. Switzerland: Springer; 2019. p. 65-99.

- Jatuwong K, Suwannarach N, Kumla J, Penkhrue W, Kakumyan P, Lumyong S. Bioprocess for production, characteristics, and biotechnological applications of fungal phytases. *Frontiers in Microbiology*. 2020;11:493778. DOI: 10.3389/fmicb.2020.493778.
- Akhtar N, Shoaib A, Munir S, Ali A, Khurshid S. Isolation, identification and enzyme production profile of *Aspergillus niger*. (In press).
- Qasim SS, Shakir KA, Al-Shaibani AB. Isolation, screening and production of phytate-degrading enzyme (phytase) from local fungi isolate. *Iraqi Journal of Agricultural Sciences*. 2016;47(1):182-92.
- Hossain, S. A., Švec, D., Mrša, V., & Teparic, R. (2018). Overview of catalytic properties of fungal xylose reductases and molecular engineering approaches for improved xylose utilisation in yeast. *Applied food biotechnology*, 5(2), 47-58.
- Corrêa TLR, de Araújo EF. Fungal phytases: from genes to applications. *Brazilian Journal of Microbiology*. 2020;51(3):1009-20. DOI: 10.1007/s42770-020-00229-w.
- Henninger C, Hoferer M, Ochsenreither K, Eisele T. Cross-linked phytase aggregates for improved phytate degradation at low pH in animal feed. *European Food Research and Technology*. 2023;249(9):2377-86. DOI: 10.1007/s00217-023-04291-1.
- Fernandes EG, Valério HM, Feltrin T, Sand STVD. Variability in the production of extracellular enzymes by entomopathogenic fungi grown on different substrates. *Brazilian Journal of Microbiology*. 2012;43(3):827-33. DOI: 10.1590/S1517-83822012000300005.
- Kumari N, Bansal S. Statistical modeling and optimization of microbial phytase production towards utilization as a feed supplement. *Biomass Conversion and Biorefinery*. 2023;13(9):8339-49. DOI: 10.1007/s13399-022-02630-8.
- Mkumbe BS, Sajidan S, Pangastuti A, Susilowati A, Greiner R. Production, characterization, and molecular phylogenetic analysis of phytase from *Aspergillus niger* isolates of an Indonesia origin. *HAYATI Journal of Biosciences*. 2022;29(4):468-78. DOI: 10.4308/hjb.29.4.468.
- Neira-Vielma AA, Aguilar CN, Ilyina A, Contreras-Esquivel JC, das Graça Carneiro-da-Cunha M, Michelena-Álvarez G, Martínez-Hernández JL. Purification and biochemical characterization of an *Aspergillus niger* phytase produced by solid-state fermentation using triticale residues as substrate. *Biotechnology Reports*. 2018;17:49-54. DOI: 10.1016/j.btre.2017.12.003.
- Mkumbe BS, Sajidan S, Pangastuti A, Susilowati A, Greiner R. Production, characterization, and molecular phylogenetic analysis of phytase from *Aspergillus niger* isolates of an Indonesia origin. *HAYATI Journal of Biosciences*. 2022;29(4):468-78. DOI: 10.4308/hjb.29.4.468.
- Hossain, S. A., Rahman, S. R., Ahmed, T., & Mandal, C. (2019). An overview of yeast cell wall proteins and their contribution in yeast display system. *Asian Journal of Medical and Biological Research*, 5(4), 246-257.
- Neira-Vielma AA, Aguilar CN, Ilyina A, Contreras-Esquivel JC, das Graça Carneiro-da-Cunha M, Michelena-Álvarez G, Martínez-Hernández JL. Purification and biochemical characterization of an *Aspergillus niger* phytase produced by solid-state fermentation using triticale residues as substrate. *Biotechnology Reports*. 2018;17:49-54. DOI: 10.1016/j.btre.2017.12.003.
- Pires EBE, de Freitas AJ, Souza FFE, Salgado RL, Guimarães VM, Pereira FA,

- Eller MR. Production of fungal phytases from agroindustrial byproducts for pig diets. *Scientific Reports*. 2019;9(1):9256. DOI: 10.1038/s41598-019-45780-w.
- Rizwanuddin S, Kumar V, Naik B, Singh P, Mishra S, Rustagi S, Kumar V. Microbial phytase: Their sources, production, and role in the enhancement of nutritional aspects of food and feed additives. *Journal of Agriculture and Food Research*. 2023;100559. DOI: 10.1016/j.jafr.2023.100559.
  - Hossain, Sk Amir, Chanchal Mandal, Toufiq Ahmed, and SM Rifat Rahman. "A REVIEW ON CONSTRUCTED GENETIC CASSETTES IN YEAST FOR RECOMBINANT PROTEIN PRODUCTION."