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, releasing inorganic phosphate that is essential for various biological processes. This study aimed to screen and characterize potential phytase-producing fungi isolated from environmental samples for extracellular phytase production. Various environmental samples were collected from different locations in the Khulna region. A total of 15 fungal isolates were obtained, and morphological and microscopic characterization aided in the preliminary identification of these fungal species. Screening for extracellular phytase production was conducted using a plate assay containing phytin as the sole phosphorus source. Five fungal isolates (Isolate 2, Isolate 3, Isolate 5, Isolate 11, and Isolate 12) yielded positive results in the phytase screening test. The total protein concentration of these five fungal isolates was determined using the TCA protein precipitation technique. Isolate 2 (8.84 µg/mL) and Isolate 12 (49.77 µg/mL) were selected for thermostability testing based on their higher protein concentrations. Both isolates exhibited positive results for thermostability, as evaluated through gradual temperature increases ranging from 4°C to 50°C. Notably, the phytase enzyme from Isolate 12 demonstrated 40% greater temperature stability compared to Isolate 2. Further studies will focus on the molecular characterization of Isolate 12 and its potential use as a feed additive. This research contributes to the discovery of potential fungal phytases with enhanced industrial applicability in various sectors.

Keywords: Enzyme Screening, ThermostablePhytase, Extracellular enzyme,Protein Precipitation, Industrial Biocatalysts

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 such as 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 by hydrolyzing phytic acid and its salts, thereby 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 phytases, particularly from fungi, being the primary enzymes used in animal feed due to their efficiency [4].

Fungal phytases, especially those produced by species such as *Aspergillusniger*, *Mucorpiriformis*, 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 demonstrating active extracellular phytase production [7].

Thermophilic fungi, in particular, have shown promise in producing thermostablephytase, 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 the 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

Environmental samples were collected from diverse regions, including various locations within the Khulna district and the Sundarbans, the largest mangrove forest in the world. The Khulna region provided samples from agricultural soils, compost piles, and forest litter, which are rich in organic matter and microbial diversity. The Sundarbans, with its unique saline and waterlogged ecosystem, served as a crucial source of fungal strains adapted to extreme environmental conditions [25]. 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 µL, 200 µL, 300 µL, 400 µL, and 500 µL 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 wash 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. Hyphal 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 insights 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 µL of PBS buffer [16].

2.4 Determination of Fungal Protein Concentration

The fungal protein concentration was determined using a NanoDrop spectrophotometer at the AcharyaPrafulla Chandra Ray Central Laboratory (APCRCL) of Khulna University, Bangladesh. The NanoDrop provided rapid and accurate measurements of protein concentration from a small 2 µL 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. In the thermostability study, we utilized a cell-free extract of the fungal protein to assess its enzymatic activity after exposure to varying temperatures. Two approaches were used, one is temperature-treated and another is non-temperature-treated samples. In the temperature-treated method, 700 µL 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 µL 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.

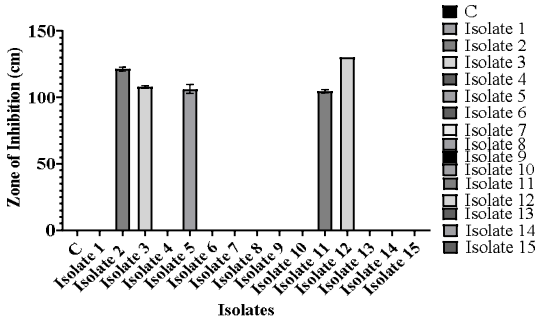

Fig. 1. Phytase Producing Fungal Isolates

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=Total diameter / Colony diameter

SE=(Total diameter / Colony diameter)×100

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

The protein concentrations of the five phytase-producing isolates were determined through extracellular fungal protein precipitation, measured using a NanoDrop spectrophotometer with two replicates for each isolate. Initial results showed that Isolate 2 exhibited protein concentrations of 9.26 µg/mL and 8.71 µg/mL, while Isolate 12 displayed 9.66 µg/mL and 9.88 µg/mL. Both isolates demonstrated higher protein concentrations compared to the other fungal strains: Isolate 3 (8.60 µg/mL and 3.35 µg/mL), Isolate 5 (9.35 µg/mL and 3.47 µg/mL), and Isolate 11 (8.70 µg/mL and 4.61 µg/mL) (Fig. 2–7).

Further analysis of three replicates revealed a significant difference between the protein concentrations of Isolate 2 and Isolate 12. Isolate 2 had relatively consistent protein concentrations of 9.79 µg/mL, 8.00 µg/mL, and 8.71 µg/mL. In contrast, Isolate 12 showed substantially higher protein concentrations at 54.72 µg/mL, 39.89 µg/mL, and 54.7 µg/mL (Fig. 8–10). The substantial increase in protein concentration in Isolate 12 compared to Isolate 2 indicates a significant difference in extracellular protein production, highlighting Isolate 12 as a superior candidate for further phytase production studies.

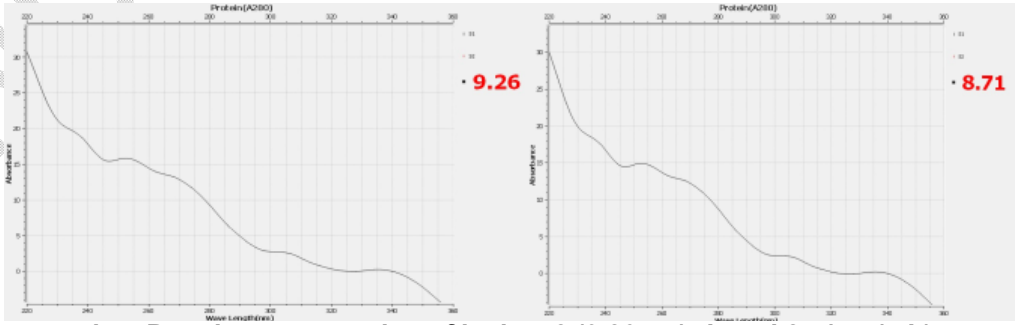

Fig.2.Protein concentration of isolate 2 (9.26 µg/mL and 8.71 µg/mL).

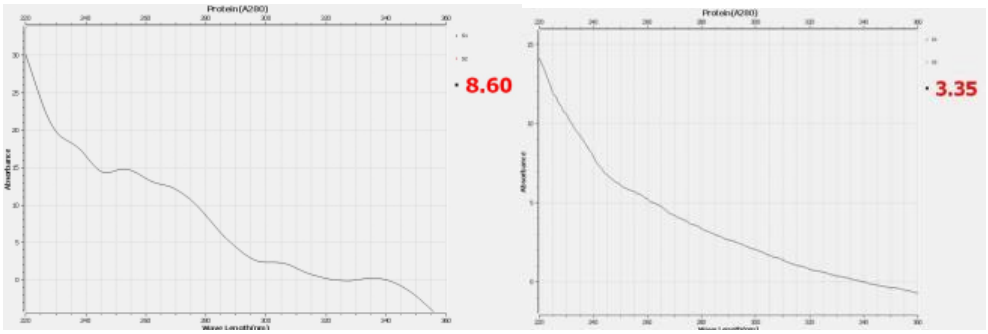

Fig.3.Protein concentration of isolate 3 (8.60 µg/mL and 3.35 µg/mL).

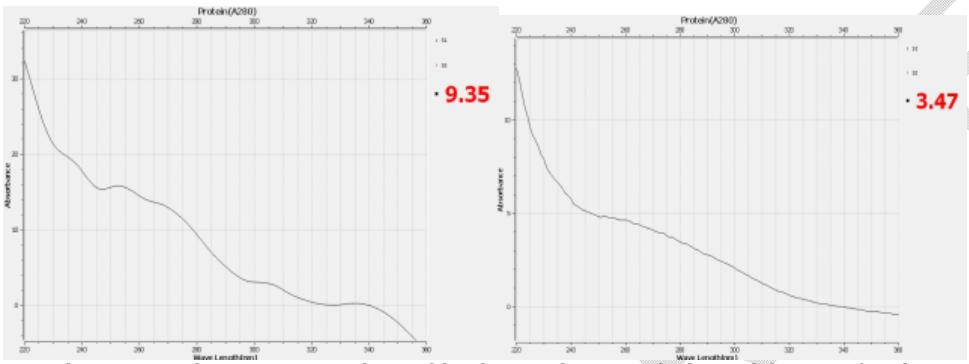

Fig.4. Protein concentration of isolate 5 (9.35 µg/mL and 3.47 µg/mL).

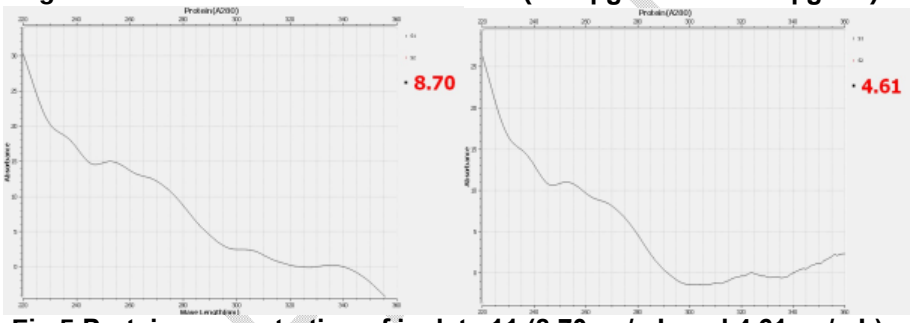

Fig.5.Protein concentration of isolate 11 (8.70 µg/mL and 4.61 µg/mL).

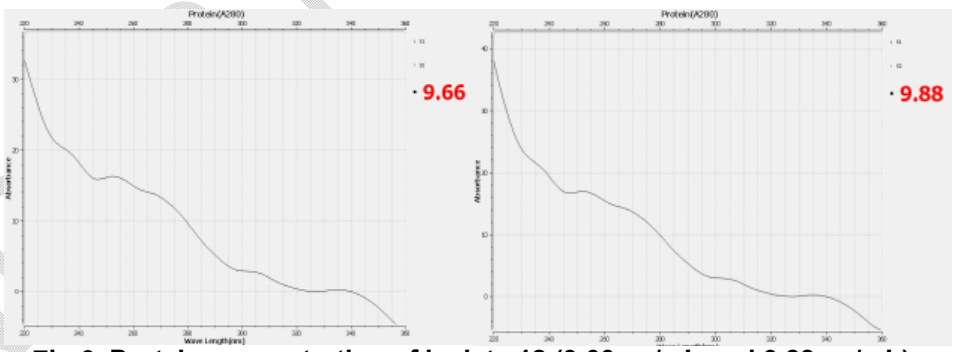

Fig.6. Protein concentration of isolate 12 (9.66 µg/mL and 9.88 µg/mL).

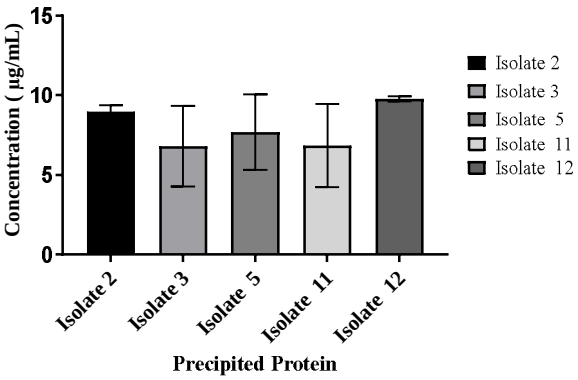

Fig.7. Total protein concentration of 5 different phytase-producing isolates.Among these five isolates, isolate 2 and isolate 12 showed a high protein concentration. Data were obtained from the extracellular fungal protein precipitation method and expressed as mean ± SD.

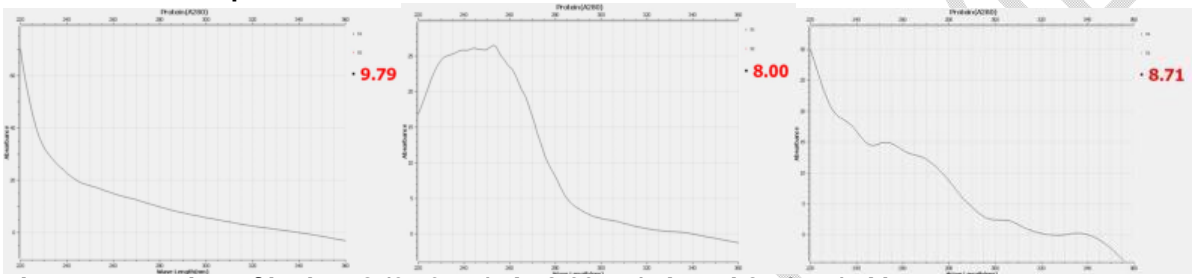

Fig.8. Protein concentration of isolate 2 (9.79 µg/mL, 8.00 µg/mL and 8.71 µg/mL).

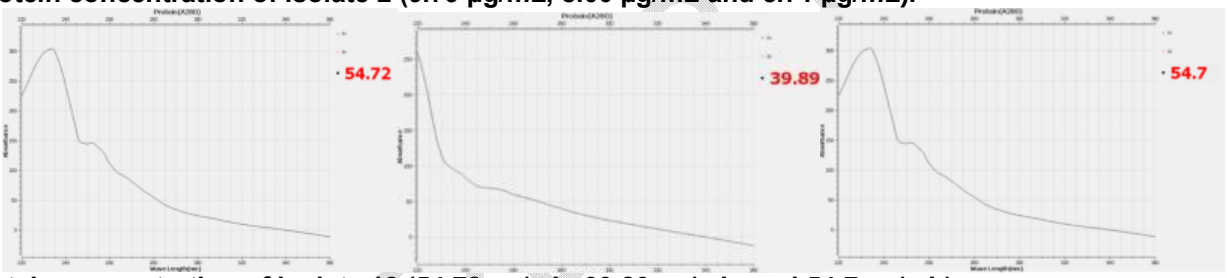

Fig.9.Protein concentration of isolate 12 (54.72 µg/mL, 39.89 µg/mL, and 54.7 µg/mL).

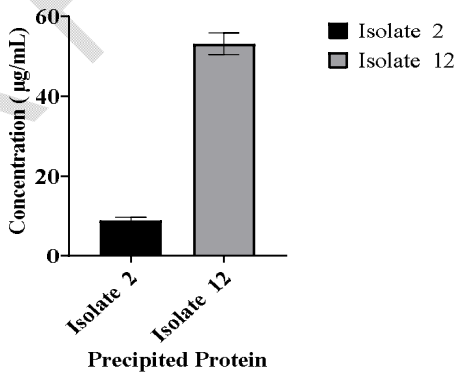

Fig.10. Total protein concentration of 2 different phytase-producing isolates.Between these two isolates, isolate 12 showed a high protein concentration. Data were obtained from the extracellular fungal protein precipitation method and expressed as mean ± SD.

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.

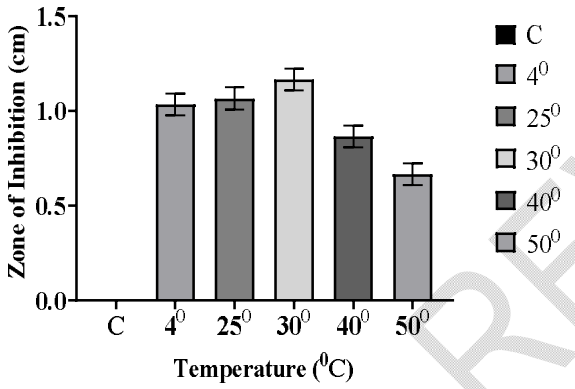

Fig.12. Temperature-dependent treatment of the thermostability test of isolate 2, conducted at 4°C, 25°C, 30°C 40°C, and 50°C, and the highest zone of inhibition appeared at 30⁰ C. Data were obtained from plate assay method and expressed as mean ± SD.

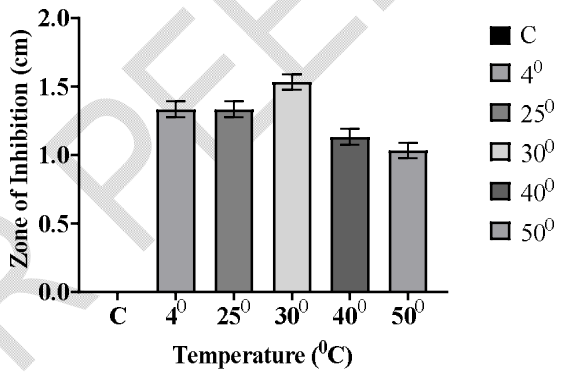

Fig.13. Temperature-dependent treatment of the thermostability test of isolate 12, conducted at 4°C, 25°C, 30°C 40°C, and 50°C, and the highest zone of inhibition appeared at 30⁰ C. Data were obtained from the plate assay method and expressed as mean ± SD.

When the isolates were tested without temperature treatment, direct inoculation of protein at 30°C, 40°C, and 50°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.

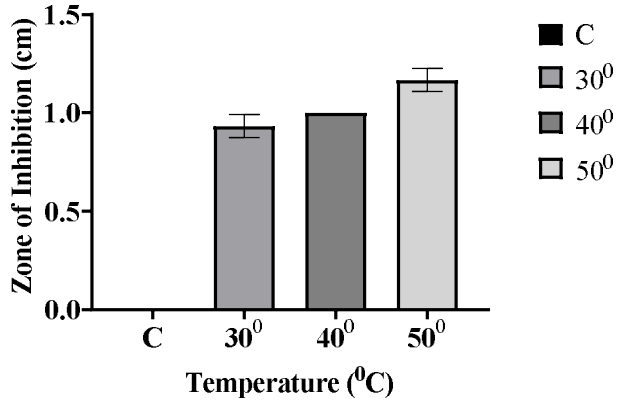

Fig.15.Thermostability test of Isolate 2 (without temperature treatment), conducted at 30°C, 40°C, and 50°C. The highest zone of inhibition appeared at 50°C. Data obtained from the plate assay method and expressed as mean ± SD.

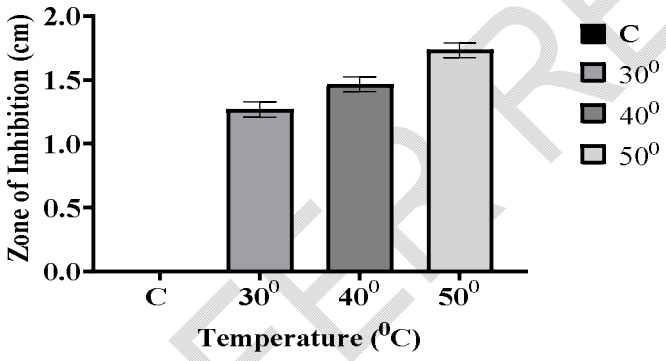

Fig.16. Thermostability test of Isolate 12 (without temperature treatment), conducted at 30°C, 40°C, and 50°C. The highest zone of inhibition appeared at 50°C. Data obtained from the plate assay method and expressed as mean ± SD.

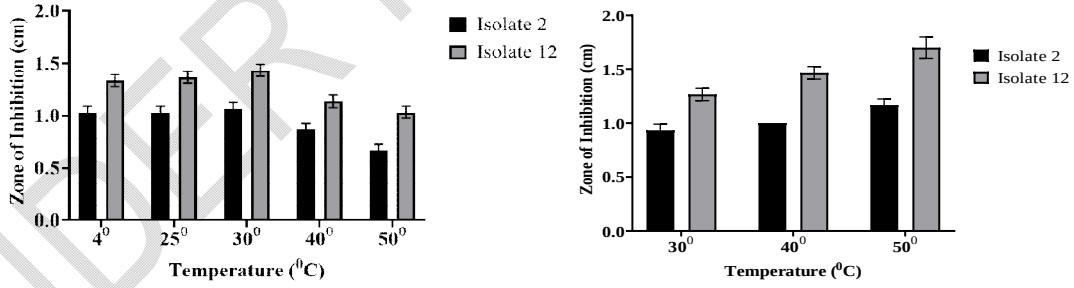

Fig.17.Comparison of Isolate 2 and Isolate 12 under temperature-treated and untreated conditions. Isolate 12 showed a higher zone of inhibition compared to Isolate 2 in both tests.

This study highlights the potential of the isolated fungal strains, particularly Isolates 2 and 12, for phytase production. Both isolates demonstrated significant zones of inhibition on phytin-enriched media under various conditions, indicating their ability to produce extracellular phytase. These findings suggest that both isolates possess thermostable properties, making them suitable for industrial applications requiring heat-resistant enzymes.Notably, Isolate 12 exhibited higher protein concentrations and larger inhibition zones than Isolate 2, both in temperature-treated and untreated conditions. This indicates that Isolate 12 not only produces more phytase but also retains its enzymatic activity better at elevated temperatures. The thermostability tests confirmed that both isolates remained active at temperatures up to 50°C, though Isolate 12 displayed a significantly greater tolerance to heat, with better performance under these conditions.

This studies have demonstrated the widespread occurrence of phytase-producing fungi in environments like soils, plant material, and organic waste [2]. Such environments provide a rich source for isolating fungi with industrial potential. The cell-free extract of fungal proteins retained significant phytase activity, as evidenced by the clear zones of inhibition observed in the plate assay. This suggests that the enzymatic components responsible for phytate degradation were effectively extracted and maintained their function outside the cellular environment. The isolation of Isolate 12, characterized by both elevated protein production and thermostability, positions it as a particularly promising candidate for industrial applications. Its superior thermostability, compared to Isolate 2, makes it highly suitable for high-temperature processes such as feed production, where heat-stable enzymes are necessary for efficient nutrient release.Further studies should explore the molecular characterization of Isolate 12 to better understand its genetic and biochemical properties. Its potential use as a feed additive for improving phosphate bioavailability in animal diets, as well as other industrial applications requiring thermostable enzymes, warrants further investigation.

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, and 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.

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