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Characterization of Endogenous Bran Phytases and Production of Stable Phytase from Mutated *Aspergillus* Species for Animal Feed Formulation

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Abstract

This research investigates the utility of enzymatic additives from both plant and microbial sources to optimize mineral uptake and growth in livestock. The study initially focused on the extraction and kinetic profiling of endogenous enzyme, specifically phytase, derived from cereal brans, followed by the purification of the plant-based phytase. Concurrently, strain improvement of Aspergillus sp. was achieved through UV irradiation and ethidium bromide mutagenesis to isolate a hyper-producing phytase variant. The microbial phytase was subsequently purified and developed into a powdered formulation. Stability assays against various chemicals and phosphorus mobilization tests confirmed the formulation's efficacy, highlighting its potential as an economical solution for animal feed supplementation.

Keywords: Strain improvement, endogenous enzymes, gel filtration chromatography, Brans.

Introduction

In the worldwide livestock industry increasingly depend on exogenous enzymatic supplementation to enhance feed efficiency and reduce environmental phosphorus runoff. In monogastric animals, such as poultry and swine, the lack of sufficient endogenous phytase prevents the breakdown of phytate, leading to poor mineral absorption and the necessity for inorganic phosphorus supplementation[1]. While cereal brans are naturally rich in enzymes like β-amylase and acid phosphatase, their native concentrations are often insufficient to meet the metabolic demands of modern high-growth breeds. Phytase catalyzes the stepwise hydrolysis of phytate into lower inositol phosphates and inorganic phosphate. By integrating plant-derived enzymes with high-activity microbial variants, producers can create a more robust enzymatic profile. *Aspergillus species* remain the gold standard for microbial production due to their high secretion capacity and the stability of their enzymes under acidic gastric conditions[2,3].

To address the high costs associated with commercial enzymes, researchers utilize mutagenesis strategies including UV irradiation and chemical agents like ethidium bromide—to develop hyper-producing strains. These "improved" microbial sources, when combined with purified plant-based extracts from cereal brans, offer a synergistic approach to mineral mobilization. This dual-source strategy not only optimizes growth parameters but also provides an economically viable alternative to expensive synthetic additives.

Materials and Methods

Chemicals: Following chemicals were used in this study: Alkaline copper reagent, DBLS, FC reagent, acid molybdate, reducing agent, DNS reagent, Sodium hydroxide, Maltose, Starch, Ammonium sulphate, Sephadex beads, potato dextrose agar, Tween 20, ethidium bromide, Sodium phytate, glucose, methanol, potassium chloride, magnesium sulphate, ferrous sulphate, Ethanol, Acetone, Glycine, Penicillin.

Extraction of phytase enzymes from brans and Kinetic assay: The extraction of endogenous enzymes was performed using a cold-solvent method to preserve catalytic stability. For phytase, 10 grams of bran were homogenized in 100 ml of 10mM acetate buffer (1:10 w/v ratio) to facilitate maximum protein solubilization, while preventing thermal degradation, the suspension was maintained in an ice bath and agitated on a magnetic stirrer at 500 rpm for 60 minutes. Following extraction, the crude slurry was clarified via centrifugation at 5000 rpm for 30 minutes at a controlled temperature of 20°C. The resulting supernatant, containing the soluble enzyme fraction, was collected for further biochemical analysis. Phytase presence was determined by measuring the activity using phytic acid as the substrate and the release of inorganic phosphate as product (Fiske-Subbarow method). Total protein estimation was conducted according to the Lowry method. All measurements were performed against appropriate reagent blanks to ensure accuracy.

Determination of Specific activity of phytase: Rate of phytase enzyme: 0.5ml of undiluted enzyme extract was pipetted out into different test tubes and the reaction was started by adding 1.0 ml of sodium phytate and the tubes were incubated for 15, 30, 45 and 60 minutes. Later the reaction was stopped by adding 1ml acid molybdate and 1ml reducing agent. Again, the tubes are incubated at room temperature for 30

minutes. Absorbance was read at 660nm using appropriate blank. A time graph was constructed using the data collected by plotting µmoles of sodium phytate as a function of time in minutes.

Estimation of protein content: 0.5 – 1.0 ml of undiluted enzyme extract is pipetted out into different test tubes and the volume is made upto 1ml with distilled water. 5ml of alkaline copper reagent was added to each test tube and incubated at room temperature for 10 minutes. Now 0.6 ml of 1:1 diluted FC reagent was added and incubated at room temperature for 30 minutes. The absorbance was read at 660nm against a suitable blank. The rate was calculated from the linear region of the time graph. Total units (IU) were calculated and hence the total protein in the given sample and the specific activity was calculated and reported.

Determination of Km and Vmax of Phytase: Different aliquots of 2 Mm was pipetted out in 10 mM acetate buffer to obtain 66.66mg$^0/_0$ to 33.33mg$^0/_0$ of substrate concentration. Volume was made up to 1.8ml with acetate buffer and the reaction was started by adding 0.5ml of suitable dilution enzyme extract. The tubes were incubated for 10 minutes and the reaction was arrested by adding 1.0ml acid molybdate and 1.0ml of reducing agent. The tubes are again incubated at room temperature for 30 minutes. The absorbance was read at 660nm against a suitable blank and graph was plotted. Km and Vmax were determined by using the Line weaver – Burk plot.

The pH optimum of Phytase: 5 ml of substrate was pipetted out into different test tubes. 1.0ml of buffers of different pH is added to those tubes. The tubes were incubated for 15 minutes after adding 1.0ml of enzyme extract. The enzyme was arrested by adding 1.0ml of acid molybdate and 1.0ml of reducing agent. The tubes are incubated for 30 minutes at room temperature. Absorbance was read at 450nm against a suitable blank. Buffers of different pH used are: Acetate buffer – 4.0, 5.0, 6.0 and 6.5, Borate buffer – 8.0 and 9.0.

Temperature optimum of Phytase: 4.0ml of substrate solution was pipetted out into different test tubes and the tubes were preincubated for 5 minutes at 10, 28, 30, 35, 40, 45, 50, 60, 70, and 90^{0}C respectively.1.0ml of buffer was added to each tube. At the end of preincubation period, the reaction was started by adding 1.0ml of enzyme to each tube. The tubes were incubated at respective temperature for 15 minutes. The reaction was arrested by adding 1.0ml of acid molybdate and 1.0ml of reducing agent. The tubes were incubated for 30 minutes at room temperature. The absorbance was read at 660nm against a suitable blank for each temperature.

Partial purification and comparative analysis of phytase activity from maize, rice bran, and sugarcane bagasse: Ammonium sulphate precipitation: 10ml of crude sample from all 3 brans are taken separately and prechilled at 4^{0}C for 10 minutes. To each of this 5.61g of ammonium sulphate was slowly added with constant stirring. This solution is allowed to stand for 15 min at 4^{0}C. Centrifuge the solution at 10,000rpm for 10 minutes. Resolve the pellet in 3ml of 10 Mm acetate buffer and the activity of phytase is checked. Finally specific activity of samples was calculated from total activity of enzyme and total protein content[4,5].

Gel filtration chromatography for the partial purification of Phytase enzyme from bran samples: Swollen gel beads of seralose 4B was equilibrated with equilibration buffer and allowed to settle in a beaker. This process is repeated until gel matrix attains equilibration. Equilibrated matrix was packed into column. After setting, 2ml of sample was loaded onto column alongside of column. Sample was allowed to sink completely into the matrix and the buffer was added. Simultaneously 1.5ml of fraction per 2minute flow rate was collected in a individual eppendorf tubes. The elution of proteins in fraction was determined spectrophotometrically by taking absorbance at 280nm. The major elition peaks observed during spectrophotometric analysis of fraction were further assayed for phytase activity and graph was plotted by taking fractions number on X-axis, activity plotted on Y-axis and protein absorbance on secondary Y-axis.

Production of Phytases by *Aspergillus* species: Isolation of mutants: The spores from 7 days old slant of *Aspergillus species* grown on Potato dextrose agar were collected by scraping in to 10ml sterile saline containing 0.01% Tween 20. After addition 0.2% ethidium bromide, the suspension was incubated at room temperature for 24hours followed by UV-irradiation, which resulted in 99% killing of spores after exposure for 4 min.

Mutants were selected by spreading UV mutated spores on slightly modified phytase screening medium (PSM) agar plates containing 0.5% sodium phytate and 0.05% sodium nitrate and selection of colony with enhancedzoneofhydrolysisbyphytase[6].

Medium and culture conditions for submerged fermentation: The fermentation medium contained (per 100 ml): Maize bran 1g; glucose 5g; Sodium nitrate 0.86g; potassium chloride 0.05g; magnesium sulphate 0.05g; ferrous sulphate 0.01g, pH 5.5 before sterilization. The above medium (100ml in 250 ml conical flask) was inoculated with 1% of spore suspension (5x10^7 spores/ml) prepared by suspending the spores from 7 day old sporulated slant of *Aspergillus* grown on potato dextrose agar in 10 ml of sterile saline containing 0.01% Tween 20 and incubated at 30°C at 200 rpm. Phytase activity was determines using modified Fiske Subbarao method.

Amount of protein and sugar in culture isolate: Protein concentration in the culture filtrate (as unknown) was determined by the method of Lowry et al. using, bovine serum albumin as a standard. Total residual reducing sugar concentration was estimated by DNS method taking culture filtrate as unknown.

Determination of stability of phytase in presence of various chemicals: To determine the stability of phytase in presence of various additives the fermented broth was supplemented with various additives like solvents (50% methanol, ethanol, glycine and acetone at room temperature), antibiotics (penicillin) for 24hrs time interval at room temperature and residual activity of phytase was evaluated[7].

Formulation of Phytase in powdered form: To evaluate the stability of phytase in powdered form the concentrated enzyme was mixed with various carriers, dried to obtain free flowing material and activity was monitored. Thus 4 ml fermented broth was mixed with 10 g sterilized carrier like wheat bran, rice bran, calcium carbonate and silica and dried at 50°C for 2 hours. Samples were removed after various time intervals and evaluated for phytase activity.

Phosphorus release by phytase in animal feed: The amount of inorganic phosphate was estimated in maize bran and then we estimated the amount of inorganic phosphate released after the addition of powder form to it and graph was plotted[8].

Results and Discussion

Extraction of phytase enzymes from brans: Extracting phytase from diverse agricultural by-products, including maize, rice, and sugarcane bagasse, reveals that these materials are significant natural reservoirs of endogenous enzymes (Figure-1). The primary goal of isolating these enzymes is to harness their potential for large-scale industrial applications.



Maize bran + Rice bran + Sugarcane bagasse = Enzyme crude

Figure-1: Extraction of phytase from maize, rice bran, and sugarcane bagasse.

Determination of Specific activity of phytases from different brans: Based on extraction and enzyme activity analysis, maize bran was identified as the most potent source of phytase. The highest specific activity of endogenous phytase was observed in maize bran (0.078 IU mg^{-1}) (Figure-2).

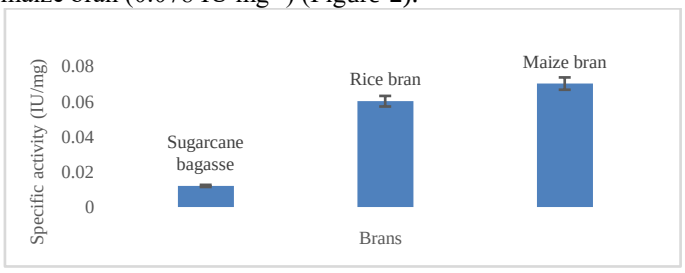


Figure-2: Specific activity of endogenous phytase in different substrates.

Determination of Km and Vmax of Phytases in brans: Kinetic parameters were determined using a Line weaver–Burk double-reciprocal plot (Figure-3). Among the substrates tested, rice bran exhibited the highest Km value (2.3 mM) and Vmax (2.1 µmol min^{-1}) for phytase.

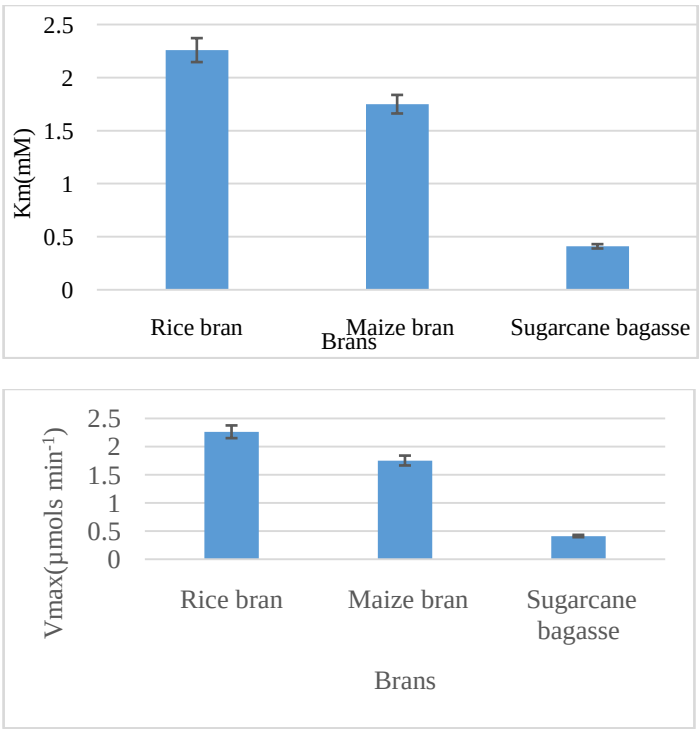


Figure-3: Kinetic parameters (Km and Vmax) of phytase in different substrates.

Determination of optimum pH of phytase from brans: The intrinsic phytases isolated from maize, rice bran, and sugarcane bagasse exhibited an optimum pH of 5.0, as shown in Figure-4.

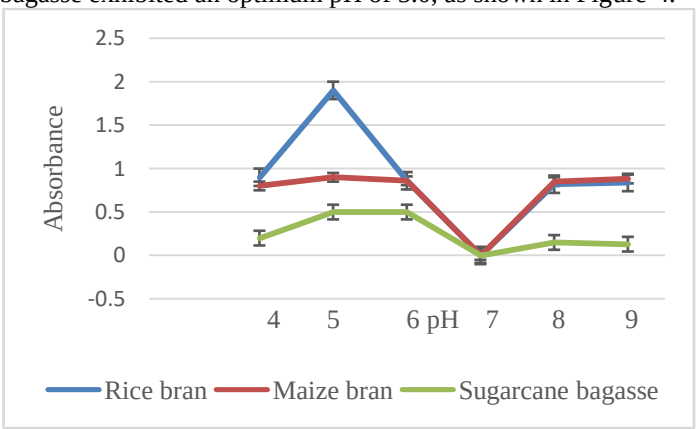


Figure-4: pH profile of phytase activity.

Determination of optimum temperature of phytase from brans: The intrinsic phytases extracted from maize, rice, and sugarcane bagasse were examined for the temperature optimum between 10-100°C. As shown in the Figure-5, the optimum temperature of phytase was found to be 80°C.

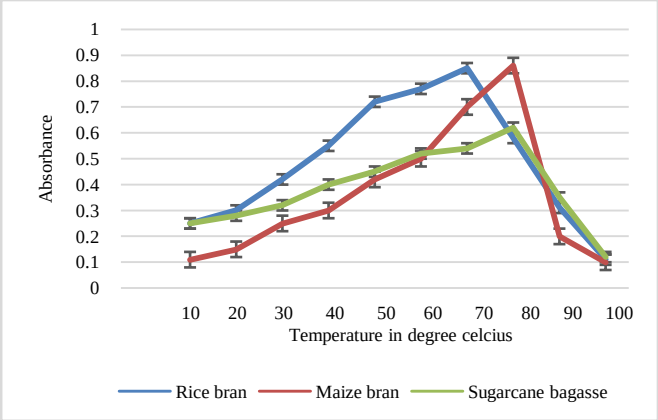


Figure-5: Effect of temperature on phytase activity.

Partial purification and comparison of Phytase enzyme activity from the bran samples (Maize, Sugarcane and Rice): Ammonium sulphate precipitation: Phytase extracted from different brans was partially purified using ammonium sulphate precipitation. Among these, sugarcane bagasse showed the highest enzyme activity (0.077 IU mg^{-1}) (Figure-6).

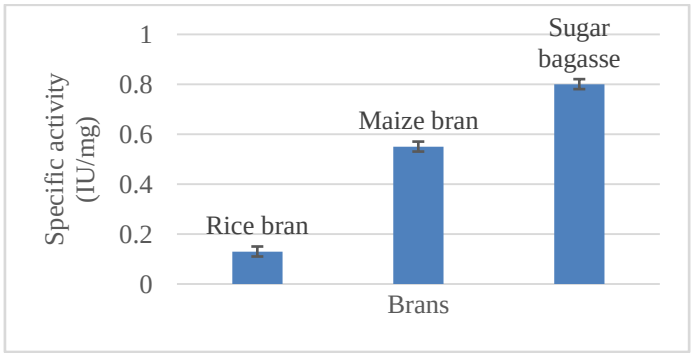


Figure-6: Activity of ammonium sulphate precipitated enzyme.

Gel filtration chromatography for the partial purification of Phytase enzyme from bran samples: Purification of phytase from different brans was carried out using gel filtration chromatography. The absorbance of the collected fractions was recorded at 280 nm, and a plot was constructed with fraction number on the x-axis and absorbance on the y-axis (Figure-7).

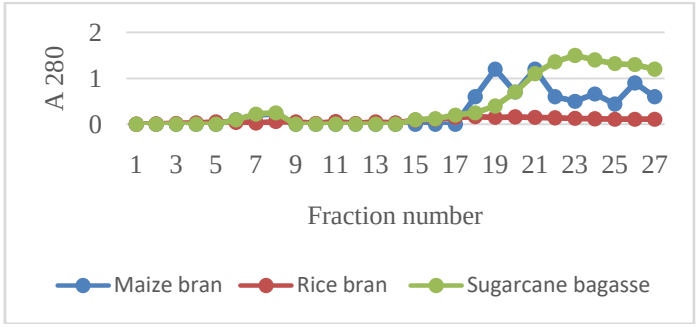


Figure-7: Elution profile of enzymes following gel filtration chromatography.

Specific activity of pooled sample from gel filtration chromatography: Fractions corresponding to the major peaks were collected, pooled, and analyzed for specific activity. The highest phytase activity was observed in maize bran (Figure-8)

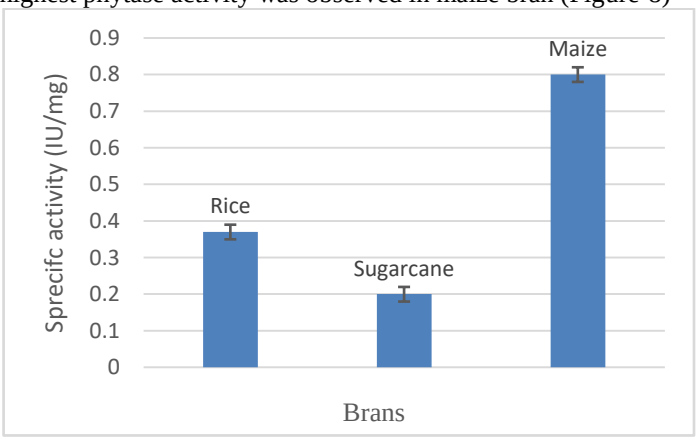


Figure-8: Specific activities of pooled fractions obtained from gel filtration chromatography.

Production of Phytases by *Aspergillus* species: Isolation of mutants and submerged fermentation: Aspergillus strains were employed for the large-scale production of phytase for industrial purposes (Figure-9A–C).

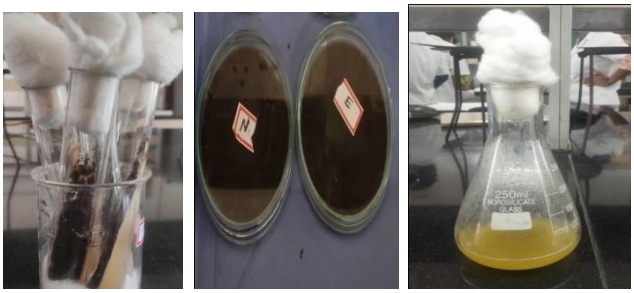
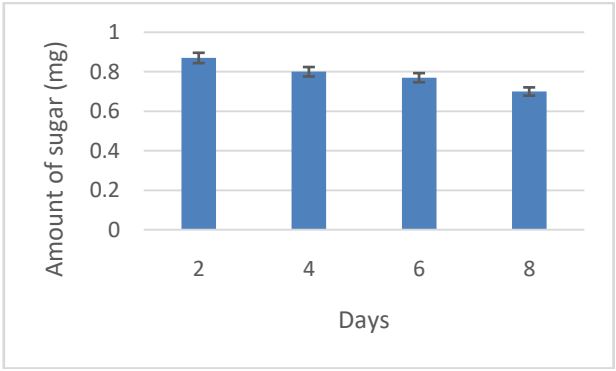


Figure-9: Mutation induction and isolation of mutants. A: Aspergillus species grown in Potato dextrose agar, B: N-Normal spores. B: E - Spores treated with Et bromide and UV radiation C: Submerged fermentation of Aspergillus using Maize bran.

Amount of protein and sugar in culture isolate: Fermentation of maize bran was carried out using *Aspergillus* species, and the residual sugar and protein content were analyzed (Figure-10).



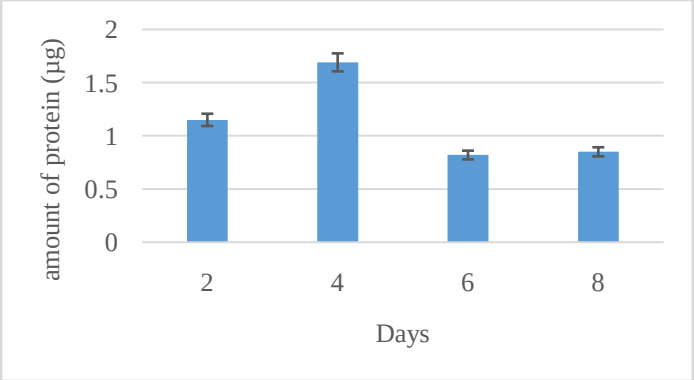


Figure-10: Changes in residual sugar and protein during fermentation.

Determination of phytase stability in the presence of various chemicals: The stability of phytase enzyme in the presence of additives is checked for 3 days and the results are checked by checking the phytase activity. Phytase enzyme was seen stable in the presence of acetone (Figure-11).

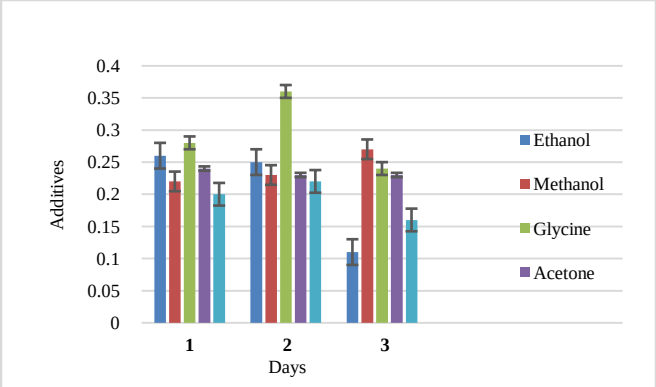


Figure-11: Effect of various chemicals on phytase stability.

Powder formulation of phytase: The purified phytase was formulated into a powdered form using appropriate carriers for industrial applications (Figure-12).



Figure-12: Powder formulated phytase.

Phosphorus release by phytase in animal feed: The powdered form of phytase was incubated with maize bran and its activity was evaluated. An increase in phosphorus content was observed following the addition of powdered phytase to maize bran used as animal feed (Figure-13).

The combination of intrinsic bran phytases and microbial mutants represents a classy approach to addressing the phosphorous utilization in monogastric animals. Many studies confirms the use of plant phytases to boost the hydrolysis of phytate. The extreme stability and high rate of production capacities of altered microbial strains are necessary to achieve industrial-scale phosphorus mobilization.

Plants are not merely substrates but also active enzymatic matrices. Wheat bran remains one of the most effective substrates for solid-state fermentation due to its natural nutrient profile[9]. Yet, research emphasizes that these native enzymes are often hindered by low thermal stability, losing significant activity during the feed pelleting process[10]. But our characterization aligns with high temperature stability phytases which can withstand processing temperature.

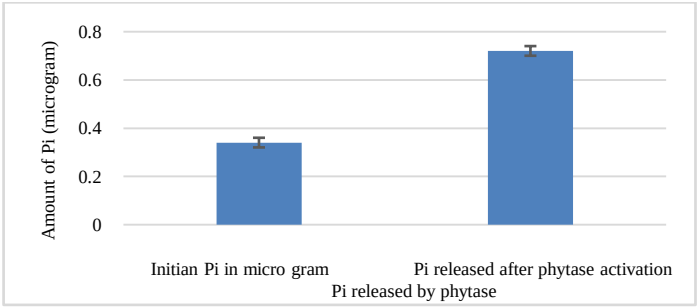


Figure-13: Effect of phytase incorporation on phosphorus release in animal feed.

The advancement of biotechnology has increasingly focused on creating robust, high-yield microbial strains through targeted mutagenesis. Research by demonstrated that specific genetic alterations within the *Aspergillus nigerphyA* gene can simultaneously enhance both the enzyme's catalytic efficiency and its resistance to heat[11]. Our application of UV radiation and ethidium bromide to induce mutations follows these established successes, aiming to develop hyper-producing strains that meet industrial demands[12].

Determining the release of inorganic phosphate from maize bran serves as a critical indicator of feed performance, as higher concentrations of phytase powder significantly improve phosphorus accessibility[13]. Our data confirms that adding mutated *Aspergillus* phytase to bran-heavy diets effectively offsets the lack of native enzymes in monogastric animals. This aligns with findings of "super-dosing" protocols enhances bone mineralization through superior nutrient uptake[14].

Conclusion

Animal feed commonly contains enzymes to enhance feed quality, which may facilitate digestion of food by animals. Brans like rice, maize and sugar cane bagasse were selected to isolate and extract the phytase. The extracted enzyme from brans was used to study the activity and kinetic parameters such

as Km and Vmax, optimum temperature and pH. These parameters are paramount in the food industry during feed manufacturing.The improved strain of *Aspergillus species* selected in phytase selecting medium by mutating with ethidium bromide and UV irradiation showed the production of phytase. The extracellular phytase produced from the *Aspergillus species* was stable in presence of various chemicals, inferring its stability with other chemicals during processing of feed by manufacturers. Phytase formulated to powdered form for industrial food manufacturing use, was showed its activity by increasing the available phosphorous in the maize bran which was selected as one of the animal feed in the current study as compared to the control. This showing the potentiality of produced phytase in increasing availability of phosphorous in the feed which helps in the quality of feed, which might boost an animal's feed intake and weight gain.

References

1. Selle, P. H., & Ravindran, V. (2007). Microbial phytase in poultry nutrition. *Animal Feed Science and Technology*, 135(1-2), 1–41.

2. Bhavsar, K., & Khire, J. M. (2014). Current status and prospects of phytase research in India. *Applied Microbiology and Biotechnology*, 98(13), 5807–5819.

3. Cao, L.; Wang, W.; Yang, C.; Yangb, Y.; Diana, J.; Yakupitiyage, A.; Luoa, Z.; Li, D. (2007). Application of Microbial Phytase in Fish Feed. *Enzyme Microb. Technol*., 40, 497–507.

4. Konietzny, U.; Greiner, R. and Jany, K.D. (1995). Purification and characterization of a phytase from spelt. *J. Food Biochem*., 18, 165-183.

5. Laboure, A.M.; Gagnon, J. and Lescure, A.M. (1993). Purification and characterization of a phytase accumulated in maize seedling during germination. *Biochem. J.*, 295, 413-419.

6. Singh, B. & Satyanarayana, T. (2011). Microbial phytases in phosphorus regeneration and agricultural application. *Applied Soil Ecology*, 47(2), 67–76.

7. Ghorbel B, Sellami-Kamoun A, Nasari M (2003). Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microbiol Technol*, 32, 513–518.

8. Greiner, R. and Konietzny, U. (2006). Phytase for food applications. *Food Technology and Biotechnology*, 44, 125-140.

9. Jatuwong, K., Suwannarach, N., Kumla, J., Penkhrue, W., Kakumyan, P., & Lumyong, S. (2020). Bioprocess for production, characteristics, and biotechnological applications of fungal phytases. *Frontiers in Microbiology*, *11*, 188.

10. Ahmad, L., Naz, S., Sultan, A., Chand, N., Islam, Z., & Khan, R. U. (2024). Effect of phytase supplementation on growth performance, mineral digestibility, and tibia characteristics in broilers. *Brazilian Journal of Poultry Science*, 26(1), 154–162.

11. Gao, Y., Zhang, W., Lan, J., Han, T., Wu, G., Wang, Y., & Luo, H. (2018). Improved thermostability and enzyme activity of a recombinant phyA mutant phytase from *Aspergillus niger* by directed evolution. *Journal of Applied Microbiology*, 124(1), 112–120.

12. Arshad, M., Ahmed, S., Abbas, M., & Shahid, M. (2023). Enhanced phytase production by *Aspergillus niger* mutants in solid state fermentation. *Kuwait Journal of Science*, 50(1A), 1–14.

13. Almeida, V., Gaffield, K. N., Kim, T. H., DeRouchey, J. M., Martin, P., Javierre, J. A., Gebhardt, J. T., Goodband, R. D., Tokach, M. D., & Woodworth, J. C. (2026). Determination of available and total tract digestible phosphorus release curves for Microtech phytase. *Journal of Animal Science and Biotechnology*, 17(2), 45–58.

14. Hakami, A. H., Al-Aqil, A. M., & Al-Khalifa, H. (2022). Efficacy and equivalency of phytase for available phosphorus in broilers fed an available phosphorus-deficient diet. *Animals (MDPI)*, 14(1), 41–55.