Partial Purification and Stability Characterisation of Phytase Produced by *Aspergillus niger* through Solid-State Fermentation of Palm Kernel Cake

Puteri Nur Fatihah R.[1], Mohd Noor Hisham, M. N.[1]*, Shamarina, S.[1], Nasyatul Ekma, M. H.[2], and Noraini, S.[2]

[1] Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[2] Livestock Science Research Centre, MARDI Headquarters, 43400 Serdang, Selangor, Malaysia

*Corresponding author: mnhisham@upm.edu.my

Received: 9 August 2026
Accepted: 27 October 2026

Abstract

Phytase produced from agro-industrial by-products may provide a sustainable enzyme source for poultry feed, but its stability requires evaluation before practical application. This study partially purified phytase produced by *Aspergillus niger* through solid-state fermentation of palm kernel cake using ammonium sulphate precipitation followed by dialysis, and characterised its storage stability, thermostability and pH stability. Among six fractions, Fraction 3 showed the numerically highest specific activity (39.743 FTU/mg), although specific activity did not differ significantly among fractions ($p = 0.370$). Storage temperature (-80, 4 and 25 °C) had no significant effect on specific activity ($p = 0.578$), whereas storage duration significantly reduced activity ($p < 0.001$). Thermostability was significantly affected by temperature, exposure time and their interaction (all $p < 0.001$); the numerically greatest retained specific activities were observed at 40 °C after 10, 20 and 30 min (30.120, 29.610 and 27.596 FTU/mg, respectively), while activity declined markedly at 80 °C. Enzyme activity was significantly affected by pH ($p = 0.003$) and remained relatively high across pH 3.0-5.5. These findings indicate favourable acidic stability but limited thermal and storage stability, suggesting that further stabilisation and in vivo evaluation are required before poultry-feed application.

Keywords: enzyme stability; fungal phytase; palm kernel cake; poultry nutrition; solid-state fermentation

Introduction

Phytate is a major storage form of phosphorus in plant-derived feed ingredients and can reduce the availability of phosphorus and other nutrients through interactions with minerals, proteins and other dietary components. Poultry have limited

capacity to hydrolyse dietary phytate; consequently, microbial phytases are widely investigated and used to improve phytate degradation and nutrient utilisation. Exogenous phytase supplementation can improve phosphorus utilisation, nutrient digestibility and indices of bone mineralisation when incorporated into phosphorus-reduced poultry diets (Moradi *et al*., 2023; Urgessa *et al*., 2024).

Beyond catalytic activity, the practical suitability of a phytase for feed-related applications depends on its physicochemical properties, including activity and stability under acidic conditions, resistance to thermal exposure during feed processing and retention of activity during storage. These characteristics should therefore be established before subsequent feed-application evaluation (Urgessa *et al*., 2024). *Aspergillus niger* is an established fungal source of phytase, although individual preparations can differ markedly in their thermal and pH characteristics. Such variation reinforces the importance of characterising each enzyme preparation rather than assuming equivalent behaviour among fungal phytases (Dotsenko *et al*., 2022).

Solid-state fermentation (SSF) provides a practical approach for producing microbial enzymes from agro-industrial residues and can contribute to the valorisation of locally available biomass. Its performance depends on factors such as substrate characteristics, moisture content, temperature, inoculum level and fermentation conditions (Perwez and Al Asheh, 2025). Palm kernel cake (PKC), a by-product of the palm oil industry, is abundant in Malaysia and has been investigated as an alternative feed and fermentation substrate (Azizi *et al*., 2021). The feasibility of phytase production from PKC by *A. niger* has previously been demonstrated under SSF conditions (Ibrahim and Lim, 2014).

However, demonstrating phytase production alone does not establish its suitability for downstream application. Information remains comparatively limited on the partial purification and stability profile of phytase recovered from PKC-based *A. niger* fermentation. Therefore, the present study aimed to (1) partially purify phytase produced by *A. niger* through SSF of PKC using sequential ammonium sulphate precipitation and dialysis, and (2) characterise the selected phytase fraction in terms of storage stability, thermostability and pH stability. These characteristics were evaluated to determine the physicochemical behaviour of the enzyme and its potential for further investigation in poultry-feed applications.

Materials and Methods

Study location

The experiments were conducted at the Feed Bioprocess Laboratory and Bioassay Laboratory, Livestock Science Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia.

Fungal culture and inoculum preparation

A pure culture of *Aspergillus niger* was obtained from the Food Technology

Culture Collection, Food Technology Research Centre, MARDI. Potato dextrose agar (PDA) was prepared at 40 g/L and sterilised at 121 °C for 15 min. The fungus was subcultured onto PDA and incubated at 30 °C for 3 d, after which the plates were maintained at 4 °C until inoculum preparation.

Spores were harvested aseptically from the sporulated cultures using sterile distilled water and counted using a Fuchs-Rosenthal haemocytometer. Serial 10^{-1} and 10^{-2} dilutions were prepared, and the working spore suspension was adjusted to 8.0×10^7 spores/mL. The same inoculum concentration was used for all SSF flasks.

Palm kernel cake and solid-state fermentation

Palm kernel cake containing <5% (w/w) shell was obtained from Felda Kernel Product Sdn. Bhd., Negeri Sembilan, Malaysia. Ten grams of PKC were placed in each fermentation flask and sterilised at 121°C at 103 kPa for 20 min before cooling to room temperature. The moisture content was maintained at 50% during fermentation. Five independent fermentation flasks were prepared.

Each flask was inoculated with 1 mL of *A. niger* spore suspension containing 8.0×10^7 spores/mL and incubated at 30°C for 3 d. Temperature and humidity were monitored throughout the incubation period.

Crude enzyme extraction

Following fermentation, each PKC preparation was mixed with 50 mL sterile distilled water for approximately 5 min. The suspension was filtered through Whatman No. 1 filter paper, and the resulting filtrate was collected as the crude enzyme preparation and stored at -80°C until partial purification.

Partial purification of phytase

The crude enzyme preparation was subjected to sequential ammonium sulphate precipitation to obtain six saturation cuts: 0-20%, 20-30%, 30-40%, 40-60%, 60-80% and 80-100% (Table 1). Ammonium sulphate was added gradually while the preparation was maintained in an ice bath and gently stirred for 1 h at each fractionation step, following the general approach described by Sandez Penidez *et al*. (2020).

Table 1: Ammonium sulphate saturation cuts used for partial purification of phytase.

Fraction	Ammonium sulphate saturation cut (%)
1	0-20
2	20-30
3	30-40
4	40-60
5	60-80
6	80-100

Following each precipitation step, the preparation was centrifuged at 5,000 rpm for 15 min. The protein pellet was resuspended in 3 mL of 0.1 mol/L acetate buffer (pH 4.5) and dialysed for 18 h against the same buffer using dialysis tubing with a 14 kDa molecular-weight cut-off. Phytase activity and protein concentration were subsequently determined for each fraction. Phytase activity and protein concentration were

assayed after dialysis and before the dialysed fractions were frozen for subsequent stability characterisation. Fraction 3, corresponding to the 30-40% ammonium sulphate saturation cut (final saturation, 40%), was selected for subsequent stability characterisation because it showed the numerically highest specific activity. Apparent activity recovery was calculated from the ratio of the mean phytase activity concentration of each fraction (FTU/mL) to the mean phytase activity concentration of the crude extract (FTU/mL) × 100. Because recovered volume was not included in this original calculation, changes associated with the 3 mL resuspension and dialysis were not used to calculate a mass-balance total FTU recovery; the values are therefore reported as apparent activity recovery.

The experiments were conducted at the Feed Bioprocess Laboratory and Bioassay Laboratory, Livestock Science Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia.

Storage stability

Fraction 3 was aliquoted into 300 µL aliquots to minimise repeated freeze-thaw cycles and stored at -80, 4 or 25°C. Phytase activity was determined from Day 0 to Day 7, and residual activity was calculated relative to the activity measured on Day 0.

Thermostability

For thermostability assessment, a 2 mL bulk preparation of Fraction 3 was pre-incubated in the digital dry-block heater for each temperature-time combination; the enzyme preparation was not subdivided into smaller thermal reaction volumes during heating. Samples were exposed at 40, 50, 60, 70 or 80°C for 10, 20 or 30 min. At each designated endpoint, the heated preparation was immediately transferred to an ice bath before phytase activity was assayed in technical triplicate.

pH stability

For pH-stability assessment, Fraction 3 was mixed at a 1:1 ratio with 0.1 mol/L buffers covering pH 2.0-8.0: KCl-HCl (pH 2.0), sodium citrate-citric acid (pH 3.0 and 6.0), sodium acetate-acetic acid (pH 4.0, 4.5, 5.0 and 5.5), and Tris-HCl (pH 7.0 and 8.0). Samples were incubated at 45 °C for 2 h, after which residual phytase activity was determined.

Phytase assay and protein determination

Phytase activity was determined using an assay adapted from Sandez Penidez *et al*. (2020). The reaction mixture contained 50 µL enzyme preparation and 200 µL of 3 mmol/L sodium phytate in acetate buffer. The mixture was incubated at 45 °C for 15 min and the reaction was stopped by adding 250 µL of 5% (w/v) trichloroacetic acid. A 100 µL aliquot of the stopped reaction was mixed with 100 µL freshly prepared colour reagent containing ferrous sulphate, ammonium molybdate and sulphuric acid, and absorbance was measured at 700 nm using an Epoch Microplate Spectrophotometer. A reagent blank in which enzyme was replaced with distilled water was processed in parallel.

Protein concentration was determined using the Bradford method (Kielkopf *et al*., 2020) according to the verified laboratory protocol. All analytical measurements were performed in technical triplicate.

Specific activity calculations

A phosphate standard curve was generated using serial dilutions of dipotassium hydrogen phosphate (K_2HPO_4). The regression equation obtained from absorbance at 700 nm was used to estimate the amount of inorganic phosphate released. Phytase activity was expressed as FTU/mL, and specific activity was calculated by dividing phytase activity by protein concentration and expressed as FTU/mg protein.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 30. Data are presented as mean ± standard error (SE). Normality was assessed before parametric analysis. Protein concentration, total phytase activity and specific activity among ammonium sulphate fractions were analysed using one-way analysis of variance (ANOVA). Storage-stability data were analysed using two-way ANOVA to determine the effects of storage temperature, storage duration and their interaction. Thermostability data were analysed using two-way ANOVA to determine the effects of temperature, exposure time and their interaction. pH-stability data were analysed using one-way ANOVA. Where significant effects were detected, means were compared using Tukey's honestly significant difference test. Statistical significance was accepted at $p < 0.05$.

Results and Discussion

Ammonium sulphate fractionation followed by dialysis produced six phytase fractions with different protein concentrations and enzyme activities (Table 2). The crude extract baseline was 1.353 ± 0.009 mg/mL protein, 29.545 ± 0.437 FTU/mL phytase activity and 21.836 ± 0.175 FTU/mg specific activity. Protein concentration and phytase activity differed significantly among fractions ($p < 0.05$), whereas specific activity did not differ significantly ($p = 0.370$). Fraction 5 showed the highest protein concentration (1.496 ± 0.008 mg/mL) and total phytase activity (48.485 ± 3.472 FTU/mL) but had the lowest specific activity (32.390 ± 1.169 FTU/mg). In contrast, Fraction 3, corresponding to the 30-40% ammonium sulphate saturation cut, showed the numerically highest specific activity of 39.743 ± 2.145 FTU/mg and a purification fold of 1.820.

The difference between total phytase activity and specific activity reflects the contribution of non-phytase proteins retained within each fraction. Consequently, a fraction with greater total protein and enzyme activity does not necessarily possess greater enzyme purity. Because specific activity among fractions was not significantly different, Fraction 3 should not be interpreted as significantly purer than the other fractions; rather, it provided the highest numerical specific activity and was

therefore selected for subsequent stability characterisation.

Purification outcomes vary with fungal strain, fermentation substrate and purification strategy. Bhandari *et al*. (2023) and Mahmood *et al*. (2024) reported different purification profiles for *A. niger* phytases using different downstream procedures. Direct numerical comparisons should therefore be made cautiously because purification procedures and assay conditions differ among studies. The modest 1.820-fold purification obtained in the present study is consistent with the limited separation expected from precipitation and dialysis alone. These relatively simple procedures concentrate protein fractions and remove low-molecular-weight components but do not provide the degree of separation achievable using chromatographic purification.

Activity recoveries exceeding 100% in Table 2 are best regarded as apparent activity recovery rather than recovery of more enzyme mass than was initially present. The values were calculated from activity concentration (FTU/mL) relative to the crude extract and did not incorporate recovered fraction volume; therefore, they do not represent total FTU mass-balance recovery. The post-dialysis phytase assay was conducted before the dialysed fractions were frozen for subsequent characterisation. Fractionation can alter measurable enzyme activity through removal of interfering components; however, this mechanism was not directly tested in the present study.

Table 2: Partial purification of phytase produced by *Aspergillus niger* through solid-state fermentation of palm kernel cake.

Fraction	**Protein concentration (mg/ml) ± SE**	**Total phytase activity (FTU/ml) ± SE**	**Specific activity (FTU/mg) ± SE**	**Purification fold**	**Apparent activity recovery (%)**
Crude Extract	1.353 ± 0.009	29.545 ± 0.437	21.836 ± 0.175	1.000	100.000
1	0.948 ± 0.011^{a}	34.343 ± 2.977^{a}	35.518 ± 3.487^{a}	1.627	116.239
2	0.992 ± 0.000^{a}	34.596 ± 1.336^{ab}	34.860 ± 1.331^{a}	1.597	117.094
3	0.998 ± 0.002^{a}	39.646 ± 2.067^{abc}	39.743 ± 2.145^{a}	1.820	134.188

4	1.230 ± 0.008^{b}	43.434 ± 2.636^{abc}	35.346 ± 2.379^{a}	1.619	147.009
5	1.496 ± 0.008^{c}	48.485 ± 3.472^{c}	32.390 ± 1.169^{a}	1.483	164.103
6	1.207 ± 0.010^{b}	47.222 ± 7.160^{bc}	39.168 ± 3.688^{a}	1.794	159.829

Values are mean ± SE. ^{abc} Different superscript letters within the same column indicate significant differences (Tukey test, $p < 0.05$). Specific activity did not differ significantly among fractions ($p = 0.370$). The Crude Extract row provides the baseline used for purification fold and apparent activity recovery calculations and was not included in the one-way ANOVA among ammonium sulphate fractions. Apparent activity recovery = (mean fraction phytase activity, FTU/mL / mean crude extract phytase activity, FTU/mL) × 100; recovered volume was not included, so these values do not represent total FTU recovery.

Storage duration significantly affected phytase specific activity ($p < 0.001$), whereas storage temperature ($p = 0.578$) and the interaction between storage temperature and duration ($p = 0.085$) were not significant (Table 3). Specific activity decreased from 32.910 ± 2.131 FTU/mg at Day 0 to 10.635 ± 1.181, 9.870 ± 0.021 and 7.088 ± 1.111 FTU/mg at Day 7 following storage at -80, 4 and 25 °C, respectively.

Relative to Day 0, the corresponding residual activities at Day 7 were 32.31, 29.99 and 21.54% at -80, 4 and 25°C, respectively. Although the numerical activity remaining at -80°C was greater than at 25°C, storage temperature did not exert a statistically significant overall effect. Therefore, the findings indicate that storage duration, rather than storage temperature within the tested range, was the dominant factor affecting enzyme activity over the seven-day period.

Phytase stability during storage can depend on enzyme origin, matrix and storage conditions. Schollenberger *et al*. (2022) demonstrated temperature- and matrix-dependent changes in intrinsic wheat phytase activity during prolonged storage, although phytase within cereal grain is not directly equivalent to the partially purified fungal enzyme examined here. More broadly, storage stability is recognised as an important criterion when evaluating microbial phytases for feed applications (Urgessa *et al*., 2024). The comparatively rapid activity decline in the present enzyme preparation indicates that storage stability remains a limitation. Strategies such as protective formulation or enzyme immobilisation may therefore warrant evaluation in future studies (Lahiji *et al*., 2021).

Table 3: Effect of storage temperature and duration on specific activity of partially purified phytase.

Day	-80 °C (FTU/mg) ± SE	4 °C (FTU/mg) ± SE	25 °C (FTU/mg) ± SE
0	32.910 ± 2.131^{a}	32.910 ± 2.131^{a}	32.910 ± 2.131^{a}
1	25.057 ± 0.478^{b}	24.297 ± 0.053^{b}	21.765 ± 0.223^{b}
2	20.250 ± 1.361^{c}	18.730 ± 0.292^{c}	19.487 ± 1.406^{c}
3	18.220 ± 0.733^{c}	18.477 ± 0.693^{c}	19.483 ± 1.745^{c}
4	15.948 ± 0.910^{cd}	15.695 ± 0.942^{cd}	18.480 ± 1.377^{cd}
5	12.903 ± 1.138^{de}	12.906 ± 1.311^{de}	15.684 ± 1.944^{de}
6	12.650 ± 0.884^{ef}	13.673 ± 1.607^{ef}	8.355 ± 0.888^{ef}
7	10.635 ± 1.181^{f}	9.870 ± 0.021^{f}	7.088 ± 1.111^{f}

Values are mean ± SE. Two-way ANOVA showed a significant effect of storage duration ($p < 0.001$), but no significant effect of storage temperature ($p = 0.578$) or temperature × duration interaction ($p = 0.085$). ^{abcdef} Different superscript letters within a column indicate significant differences among storage days (Tukey test, $p < 0.05$).

Temperature, exposure time and the temperature × time interaction significantly affected phytase specific activity (all $p < 0.001$; Table 4). The numerically greatest retained activities were observed at 40°C, with specific activities of 30.120 ± 1.286, 29.610 ± 0.377 and 27.596 ± 2.127 FTU/mg following exposure for 10, 20 and 30 min, respectively.

After 10 min of exposure, activities at 40-70°C were not clearly separated statistically, whereas activity was lower at 80°C. The effect of thermal exposure became more pronounced with longer incubation. At 80°C, specific activity declined from 20.502 ± 0.481 FTU/mg after 10 min to 12.909 ± 1.325 FTU/mg after 20 min and 5.056 ± 1.327 FTU/mg after 30 min. The corresponding residual activities were 62.30, 39.22 and 15.36%, respectively. These results demonstrate a clear temperature-time dependency rather than a single absolute thermal threshold. Thermostability describes retention of enzyme activity after heat exposure and should not be interpreted as the optimum reaction temperature.

Thermal behaviour varies considerably among fungal phytases. Differences reported for *A. niger* phytases may reflect fungal strain, protein structure, glycosylation, purification procedure and assay conditions (Bhandari *et al*., 2023; Dotsenko *et al*., 2022; Mahmood *et al*., 2024). Thermal resistance is particularly relevant to feed enzymes because they may encounter elevated temperatures during feed processing. Protein engineering and other

stabilisation approaches have therefore been explored to improve phytase thermostability (Tu *et al*., 2024; Venkataraman et al., 2025). The present unformulated partially purified phytase exhibited limited resistance to prolonged high-temperature exposure, particularly at 80°C, indicating that thermal protection or alternative application strategies should be evaluated before feed-processing applications are considered.

Table 4: Effect of temperature and exposure time on specific activity of partially purified phytase.

Temperature (°C)	**10 min (FTU/mg) ± SE**	**20 min (FTU/mg) ± SE**	**30 min (FTU/mg) ± SE**
40	30.120 ± 1.286^{ax}	29.610 ± 0.377^{ax}	27.596 ± 2.127^{ax}
50	27.834 ± 1.934^{ax}	24.546 ± 1.060^{bx}	24.295 ± 0.388^{ax}
60	26.322 ± 1.114^{ax}	24.297 ± 0.455^{bxy}	22.523 ± 0.652^{ay}
70	25.057 ± 0.893^{abx}	21.012 ± 1.380^{bx}	16.199 ± 0.686^{by}

Note: Values are mean ± SE. Temperature, exposure time and their interaction were significant (all $p < 0.001$). Within a column, ^{abc} different superscript letters indicate significant differences among temperatures; within a row, ^{xyz} different superscript letters indicate significant differences among exposure times (Tukey test, $p < 0.05$).

Specific activity differed significantly among pH conditions ($p = 0.003$; Table 5). Activity remained comparatively high from pH 3.0 to 5.5, with the numerically highest activities recorded at pH 5.0 (26.318 ± 0.975 FTU/mg) and pH 5.5 (26.320 ± 0.649 FTU/mg). The lowest activity was recorded at pH 8.0 (11.640 ± 0.902 FTU/mg).

Residual activity followed a similar pattern, with approximately 73.86-79.98% activity retained between pH 3.0 and 5.5, compared with 35.37% at pH 8.0. Conditions at pH 2.0, 6.0 and 7.0 were intermediate. This acidic stability profile is broadly consistent with several microbial and fungal phytases, although the precise pH characteristics vary among strains and preparations (Bhandari *et al*., 2023; Mahmood *et al*., 2024; Nascimento *et al*., 2022).

Acid stability is relevant to potential phytase use in poultry nutrition because enzyme activity must be retained under acidic conditions in the upper gastrointestinal tract. Reviews of microbial phytases identify acidic pH activity, thermal stability and storage stability as important characteristics when screening candidate enzymes for poultry-feed application (Nezhad *et al*., 2020; Urgessa *et al*., 2024). Nevertheless, the present pH-stability experiment was conducted *in vitro.* The ability of the

partially purified phytase to retain relatively high activity between pH 3.0 and 5.5 therefore supports further evaluation under simulated gastrointestinal and poultry-feeding conditions but does not by itself demonstrate *in vivo* efficacy.

Table 5: Effect of pH on specific activity of partially purified phytase.

pH	Specific activity (FTU/mg) ± SE
2.0	19.999 ± 1.143^{ab}
3.0	24.307 ± 2.778^{a}
4.0	23.543 ± 1.363^{a}
4.5	23.528 ± 2.224^{a}
5.0	26.318 ± 0.975^{a}
5.5	26.320 ± 0.649^{a}
6.0	22.000 ± 4.303^{ab}
7.0	19.735 ± 1.720^{ab}
8.0	11.640 ± 0.902^{b}

Values are mean ± SE. One-way ANOVA showed a significant pH effect ($p = 0.003$). [ab] Different superscript letters indicate significant differences among pH levels (Tukey test, $p < 0.05$).

Conclusion

Phytase produced by *Aspergillus niger* through solid-state fermentation of palm kernel cake was successfully partially purified using sequential ammonium sulphate precipitation and dialysis. Fraction 3, representing the 30-40% ammonium sulphate saturation cut, showed the numerically highest specific activity (39.743 FTU/mg), although specific activity did not differ significantly among the six fractions. Characterisation of Fraction 3 demonstrated that storage duration significantly reduced enzyme activity irrespective of storage temperature, while thermostability decreased progressively with increasing temperature and exposure time, particularly at 80°C. In contrast, the enzyme retained relatively high activity under acidic conditions between pH 3.0 and 5.5. Collectively, these findings demonstrate favourable acidic stability but limitations in storage and high-temperature stability. Further studies should evaluate appropriate stabilization strategies, phytate-hydrolysis efficiency in feed matrices, gastrointestinal stability and in vivo efficacy before the enzyme is considered for practical poultry-feed application.

Acknowledgement

The authors gratefully acknowledge the Malaysian Agricultural Research and Development Institute (MARDI), particularly the Livestock Science Research Centre, and the Faculty of Science, Universiti Putra Malaysia, for providing laboratory facilities and technical support for this study.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Azizi, M. N., Loh, T. C., Foo, H. L., & Chung, E. L. T. 2021. Is palm kernel cake a suitable alternative feed ingredient for poultry? *Animals* 11(2): 338.

Bhandari, Y., Sonwane, B., & Vamkudoth, K. R. 2023. Isolation and biochemical characterization of acid phytase from *Aspergillus niger* and its applications in dephytinization of phytic acid in poultry feed ingredients. *Microbiology* 92(2): 221-229.

Dotsenko, A., Rozhkova, A., Zorov, I., Korotkova, O., & Sinitsyn, A. 2022. Enhancement of activity and thermostability of *Aspergillus niger* ATCC 10864 phytase A through rational design. *Biochem. Biophys. Res. Commun.* 634: 55-61.

Ibrahim, D., & Lim, S. H. 2014. Utilization of palm kernel cake as a novel substrate for phytase production by *Aspergillus niger* USM AI1. *BioResources* 9(1): 1488-1497.

Kielkopf, C. L., Bauer, W., & Urbatsch, I. L. 2020. Bradford assay for determining protein concentration. *Cold Spring Harb. Protoc*. 2020(4): pdb.prot102269.

Lahiji, S., Hemmati, R., Homaei, A., Saffar, B., & Ghorbani, M. 2021. Improved thermal stability of phytase from Yersinia intermedia by physical adsorption immobilization on amino-multiwalled carbon nanotubes. *Bioprocess Biosyst*. *Eng*. 44(10): 2217-2228.

Mahmood, S., Shahid, M. G., Nadeem, M., Nelofer, R., & Irfan, M. 2024. Purification and characterization of phytase produced from *Aspergillus niger* using solid state fermentation. *Cellulose Chem. Technol.* 58(9-10): 1091-1098.

Moradi, S., Abdollahi, M. R., Moradi, A., & Jamshidi, L. 2023. Effect of bacterial phytase on growth performance, nutrient utilization, and bone mineralization in broilers fed pelleted diets. *Animals* 13(9): 1450.

Nascimento, J. C. S., Ribeiro, A. G., Pessoa, R. A. S., Rabello, C. B. V., Venâncio, A., Porto, T. S., Teixeira, J. A. C., & Porto, A. L. F. 2022. Effect of pH and temperature on phytase and biomass production by submerged fermentation with *Aspergillus niger* var. phoenicis URM 4924. *Res. Soc. Dev*. 11(6): e41311628994.

Nezhad, N. G., Rahman, R. N. Z. R. A., Normi, Y. M., Oslan, S. N., Shariff, F. M., & Leow, T. C. 2020. Integrative structural and computational

biology of phytases for the animal feed industry. *Catalysts* 10(8): 844.

Perwez, M., & Al Asheh, S. 2025. Valorization of agro-industrial waste through solid-state fermentation: Mini review. *Biotechnol.* Rep. 45: e00873.

Sandez Penidez, S. H., Velasco Manini, M. A., Gerez, C. L., & Rollán, G. C. 2020. Partial characterization and purification of phytase from Lactobacillus plantarum CRL1964 isolated from pseudocereals. *J. Basic Microbiol*. 60(9): 787-798.

Schollenberger, M., Sommerfeld, V., Hartung, J., & Rodehutscord, M. 2022. Storage duration and conditions change the phytate content and phytase activity of wheat grains. *JSFA Rep*. 2(3): 100-106.

Tu, T., Wang, Q., Dong, R., Liu, X., Penttinen, L., Hakulinen, N., Tian, J., Zhang, W., Wang, Y., Luo, H., Yao, B., & Huang, H. 2024. Achieving thermostability of a phytase with resistance up to 100 °C. *J. Biol. Chem*. 300(12): 107992.

Urgessa, O. E., Koyamo, R., Dinka, H., Tefese, K., & Gemeda, M. T. 2024. Review on desirable microbial phytases as a poultry feed additive: Their sources, production, enzymatic evaluation, market size, and regulation. *Int. J. Microbiol*. 2024: 9400374.

Venkataraman, S., Karthikanath, P. R., Gokul, C. S., Adhithya, M., Vaishnavi, V. K., Rajendran, D. S., Vaidyanathan, V. K., Natarajan, R., Balakumaran, P. A., & Kumar, V. V. 2025. Recent advances in phytase thermostability engineering towards potential application in the food and feed sectors. *Food Sci. Biotechnol.* 34(1): 1-18.