



Research Article

Effect of Proteolytic Enzymes on Phytase Produced by *Pseudomonas spp.* Isolated from Local Chicken Faeces

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ABSTRACT

Phytases are special types of phosphatases that catalyze the sequential hydrolysis of phytic acid to less phosphorylated myo-inositol derivatives and inorganic phosphate. Phytic acid is one of the main storage forms of phosphorus in plants. This study is aimed at investigating the effect of proteolytic enzymes on phytase activity. Standard methods were used during the investigations. The effects of proteolytic enzymes and gastric acid on crude phytase enzyme were studied. Phytase enzyme kinetics (K_M and V_{max}) were also determined using varying substrate concentrations. The results revealed that optimum phytase activity ($0.53 \mu\text{mol}/\text{min}$) was obtained when the crude phytase enzyme was treated with trypsin at 6.0% (v/v) while treatment with pepsin yielded highest activity ($1.07 \mu\text{mol}/\text{min}$) at 6.0% (v/v). A lower phytase activity was observed upon treatment with gastric acid. The double reciprocal graph of substrate concentration revealed that the K_M and V_{max} of phytase were 0.0029 mM and $3.744 \mu\text{mol}/\text{min}$ respectively. It could be concluded that the study demonstrated that crude phytase from *Pseudomonas spp.* retained measurable activity in the presence of proteolytic enzymes. These findings suggest potential suitability of the enzyme for application in animal nutrition.

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INTRODUCTION

Phytases have received a lot of interest recently from researchers and entrepreneurs especially those working in the fields of biotechnology, environmental protection, and nutrition (Herrmann *et al.*, 2021). In order to endure the feed pelleting process, phytases, (industrially significant feed enzymes) underwent extensive thermostability engineering (Herrmann *et al.*, 2021). Additionally, several naturally occurring phytases were modified through genetic engineering to have greater pH stability or increased specific activity in order to effectively hydrolyze phytate in the digestive tract of monogastric animals (Herrmann *et al.*, 2019). These enzymes belong to a special class of phosphomonoesterases (myo-inositol hexakisphosphate 3-phosphorylase, EC 3.1.3.8 and myo-inositol hexakisphosphate 6-phosphorylase, EC 3.1.3.26), and are capable of initiating the sequential release of phosphate from phytate (myo-inositol hexakisphosphate), which is considered the major storage form of phosphate in plant seeds and pollen (Blaabjerg *et al.*, 2016). Phytases were originally proposed as an animal feed additive to improve the nutritional quality of plant material in feed for monogastric animals by releasing phosphate (Mitchell *et al.*, 2017). A comprehensive approach to phosphorus management encourages breaking the cycle of this nutrient's depletion. As a result, various amounts of modified phytases support a sustainable bioeconomy (Infanzón *et al.*, 2022). Furthermore, phosphorus recovered from renewable resources like deoiled seeds causes both phosphorus deficient feed and a decrease in bioavailability in animal feed. (Herrmann *et al.*, 2020). This decreases phosphorus accumulation in the fields through the use of phytate-poor manure. A biotechnological use of phytase in the food industry was considered since phytate acts as an antinutrient by attaching to proteins and chelating minerals (Rousseau *et al.*, 2012). Phytic acid is one of the main storage forms of phosphorus in plants. Although, phytic acid is present in soil as plant based organic matter, it is unavailable for plants (Lazali *et al.*, 2013; Tso *et al.*, 2020). Due to its unusual molecular structure, phytate has the ability to form complexes with other nutrients such as metallic ions (Mg^{2+} , Ca^{2+} , Fe^{2+} , Cu^{2+} etc) rendering them unavailable to plants (Selle *et*

al., 2012). Due to its high phosphate concentration, phytic acid is different from other organo-phosphates and has a structure that is very stable (Kerovuo *et al.*, 2020).

Plant seeds with similar phytate levels include cereals (maize, barley, wheat, and millet) and legumes that are frequently used as feed components. Phytate makes up, on average, 70% of the total phosphorus present in the feed ingredients (Maenz, 2019). The intestines of monogastric animals (such as pigs, poultry, and fish) lack phytase activity and are therefore unable to utilize phosphorus from phytic acids. As a result, feed must be supplemented with inorganic phosphate. Therefore, the manure secreted by animals contain high amount of phosphorus that may cause environmental pollution, eutrophication of surface water, particularly in fields of intensive livestock production. Phytate is known as an antinutritional factor, since it chelates various metal ions such as Mg^{2+} , Ca^{2+} etc and forms complex with proteins (Maenz, 2019).

Several strains of bacteria, yeasts and fungi have been used for production of phytase on large scale for commercial purposes. Many bacterial strains have been studied for the phytase production such as *Pseudomonas spp*, *Bacillus spp*, *Raoultella spp*, *Escherichia coli*, *Citrobacter braakii* etc and fungal strains such as *Aspergillus niger*, *Thermomyces lanuginosus*, *Aspergillus terreus* etc (Selvamohan *et al.*, 2012). Phytase production is widely affected by medium composition used for bacterial or fungal culture and the production is mainly induced by nutrient and physical conditions (Infanzón *et al.*, 2022). Phytases have been mainly used for swine and poultry and also fish. The first commercial phytase products were launched into the market in 1991, and now the market volume is up to 150million dollar (Infanzón *et al.*, 2022). Bulama *et al.* (2017) reported that phytase relative enzyme activity shows that the enzyme preparation has half-life of about 87 min indicating that the enzyme is thermostable. Despite the wide application of phytases in animal nutrition, limited information exists regarding the stability of microbial phytases in the presence of digestive proteolytic enzymes such as pepsin and trypsin. These enzymes play important roles in protein digestion within the gastrointestinal tract of monogastric animals and may

influence the functional stability of phytase during digestion. Understanding the interaction between phytase and digestive proteases is therefore important for evaluating the suitability of phytase as a feed additive. Therefore, the present study aimed to investigate the effect of proteolytic enzymes (pepsin and trypsin) and gastric acid on phytase activity.

Materials and Methods

Sample Collection and processing

Local chicken faeces were collected from *Yankaji* located at Monday Market Maiduguri, Borno State, Nigeria in a closed container. The sample (local chicken faeces) was then taken to Department of Crop Protection, Faculty of Agriculture for processing. One gram of local chicken faeces was suspended in 9 mL of sterile normal saline to obtain the stock suspension. Serial dilutions were prepared by transferring 1 mL of the suspension into 9 mL normal saline to obtain dilutions ranging from 10^{-1} to 10^{-6} . 0.1 mL from appropriate dilutions were spread onto nutrient agar plates and incubated at 37°C for 24 h for microbial growth. Microbial growth was estimated using colony counter.

Isolation and Identification of the Microorganism

Bacterial specie (*Pseudomonas spp.*) identification was subjected to the method described by Samson, (2001). The serially diluted sample was cultured in a petri-dish containing solid media (nutrient agar) to allow microbial growth. Each distinct colony was subcultured and incubated for 24 hours. The colonies obtained were gram stained using gram staining method.

Assessing the Phytase Production Potential of the Microbial Specie

Phytase production was an overnight seed culture of phytase producing microorganism (*Pseudomonas spp.*) prepared by cultivating the strain in a petri-dish containing nutrient agar. Afterwards, 10% of the seed culture was inoculated into 50 ml of production medium containing K_2HPO_4 (0.1%) (w/v), $NaCl_2$ (1%) (w/v), $MgSO_4 \cdot 7H_2O$ (0.01%) (w/v) and peptone (0.5%) (w/v). Incubation was carried out at 37°C for 72 hours. All the experiments were done in triplicate and average values were recorded. After 72 hours of incubation the cells were harvested by centrifugation at 10,000 rpm for 15 minutes and the supernatant was used as an enzyme source for estimation

of phytase activity (Singh and Satyanarayana, 2008).

Assay of Phytase Activity

Phytase activity was assayed using the method described by Singh and Satyanarayana (2008). To the 0.2 ml of the crude enzyme sample, 2.4 ml phytic acid solution (0.32 g Sodium phytate, dissolved in 50 ml of 0.2 M sodium acetic acid buffer with pH 5.5) was added. To this mixture, 1ml of 0.1 M $MgSO_4 \cdot 6H_2O$ and 0.4 ml of distilled water was added. The content was incubated at room temperature for 15 minutes and the reaction was arrested with the addition of 0.5 ml Trichloroacetic acid (10%) (w/v) and after that 1 ml of distilled water and 2.5 ml of Taussky-schoor reagent was added to this mixture, test tubes containing only reagents and distilled water were used as blanks, A control sample containing phytase without proteolytic enzyme treatment was incubated under identical experimental conditions and used as a reference for comparison of enzyme activity. the absorbance was measured at 660 nm using spectrophotometer. Phytase activity was calculated using phosphorus standard. One (1) unit of phytase activity is equivalent to $1\mu\text{mol}$ of phosphorus released under standard assay conditions.

Effect of Proteolytic Enzymes on Phytase

Pepsin – 0.5ml of pepsin solution (10mg pepsin in 0.2M Glycine-HCl buffer pH 2.5) was added to 0.1ml of phytase diluted in 2.5ml 100mM sodium buffer (pH 5.5), then the pH of the mixture was adjusted to pH 2.5 with 0.5M HCl. The content was incubated at room temperature for 15 minutes.

Trypsin – 0.5ml of trypsin solution (10mg trypsin in 0.2M Glycine-HCl buffer pH 2.5) was added to 0.1ml of phytase diluted in 2.5ml 100mM sodium buffer (pH 5.5), then the pH of the mixture was adjusted to pH 2.5 with 0.5M HCl. The content was incubated at room temperature for 15 minutes.

Gastric acid – Gastric acid solution was prepared by mixing 0.5 M HCl, 0.2 M $NaHCO_3$, pepsin (10 mg/mL), and trypsin (10 mg/mL) in 0.2 M Tris-maleate buffer (pH 6.8). The solution was freshly prepared prior to the experiment. 0.5ml of gastric acid solution was added to 0.1ml of phytase diluted in 2.5ml of 100mM sodium acetate buffer (pH 5.5) after the pH of the mixture was adjusted to pH 6.8 with 0.5M NaOH (Boyce and Wals 2006). The content was incubated at room temperature for

15 minutes. Phytase activity was assayed as per the method described earlier (Sasirekha *et al.*, 2012).

Phytase Enzyme Kinetics Determination

By evaluating the rate of phytate hydrolysis in the standard reaction mixture (at 37°C and pH 6.0 sodium acetic acid buffer) at varying substrate (phytic acid) concentrations ranging from 0.2 to 6 mM, the kinetic parameters of phytase were determined. The Lineweaver-Burk plot was used to calculate the Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) values using Microsoft Excel.

Statistical Analysis

Results are expressed as mean \pm Standard Error of Mean (SEM). Differences among the mean of the groups was determined with one-

way analysis of variance (ANOVA) using SPSS software package 21.0. P values < 0.05 were considered significant.

Results

Effect of Proteolytic Enzymes on Phytase

Optimal phytase activity (0.53 $\mu\text{mol}/\text{min}$) was obtained when treated with pepsin at 6.0% while lowest activity (0.28 $\mu\text{mol}/\text{min}$) was observed when treated with 0.5% of pepsin (Figure 1.0). Treatment with trypsin yielded highest activity (1.07 $\mu\text{mol}/\text{min}$) at 6.0% and its lowest activity (0.61 $\mu\text{mol}/\text{min}$) was observed at 0.5% (Figure 1.1). The increase in phytase activity following treatment with pepsin and trypsin was statistically significant ($p < 0.05$) compared with the control.

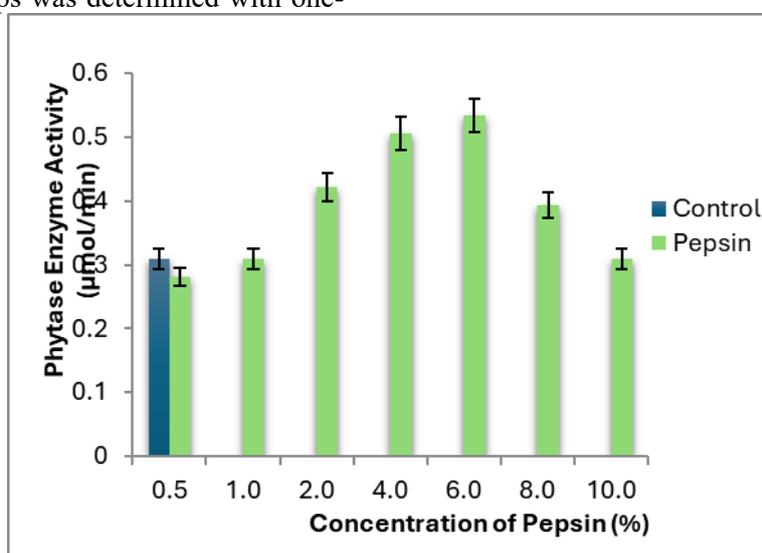


Fig. 1.0: Effect pepsin on phytase

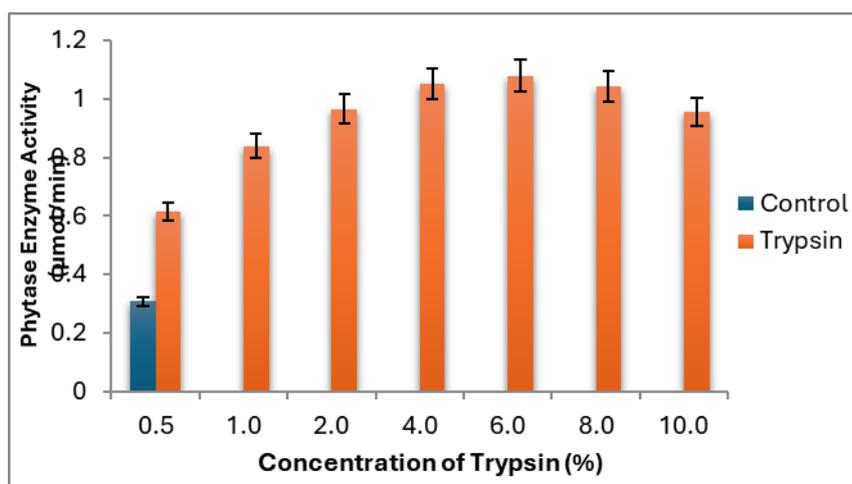


Fig. 1.1: Effect trypsin on phytase

Effect of Gastric Acid on Phytase

Maximum phytase activity ($0.24 \mu\text{mol}/\text{min}$) was observed when treated with 8.0% of gastric acid and the lowest activity ($0.21 \mu\text{mol}/\text{min}$) was obtained at 2.0% (Figure 1.2).

The increase in phytase activity following treatment with gastric acid was statistically significant ($p < 0.05$) compared with the control.

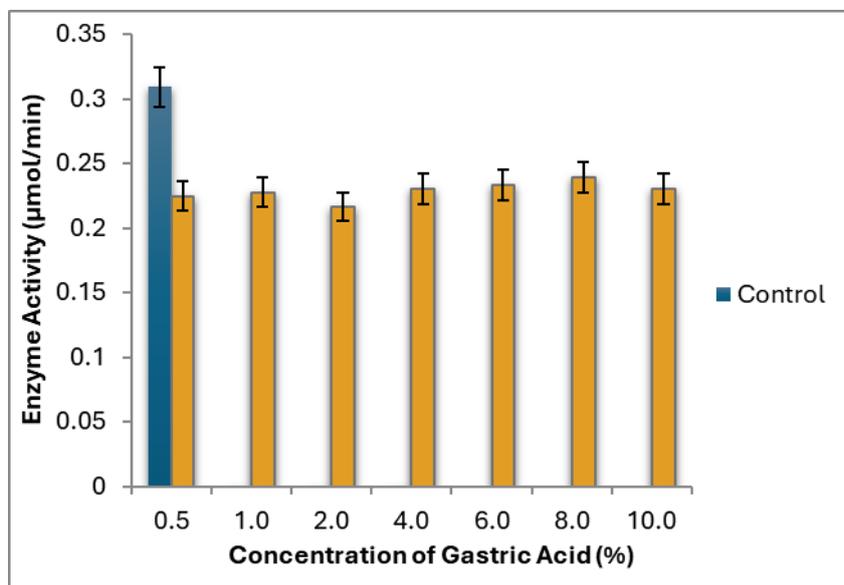


Fig. 1.2: Effect gastric acid on phytase

Phytase Enzyme Kinetics

The graph revealed that the maximum velocity (V_{max}) was $3.744 \mu\text{mol}/\text{min}$ and the K_M value was 0.0029 mM (Figure 1.3).

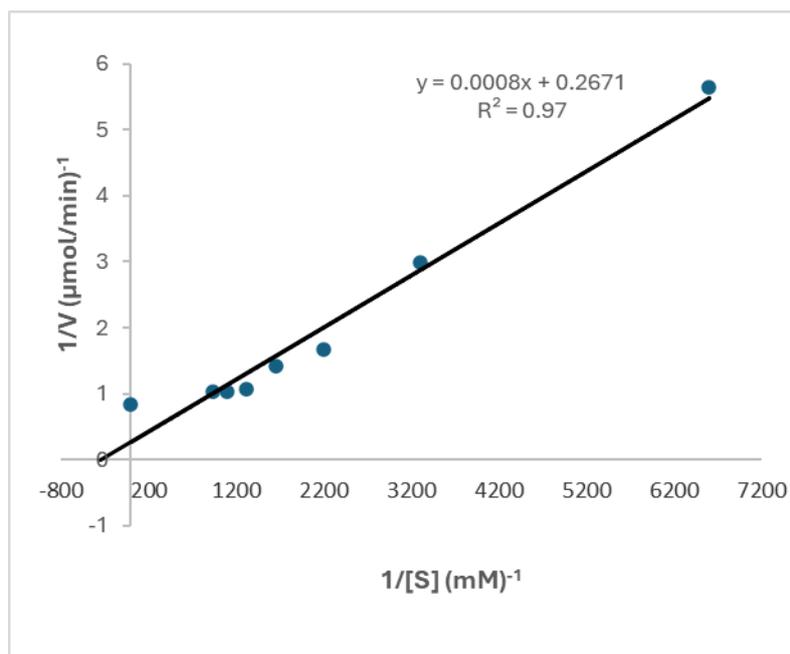


Fig. 1.3: Phytase enzyme kinetics

DISCUSSION

The effects of proteolytic enzymes on proteins are well understood. Results of pepsin's effect on phytase showed that when pepsin was used as a treatment, phytase activity was increased (Figure 1.0). Phytase activity was at its peak after being exposed to 6.0% of pepsin. The results of Greiner *et al.* (2019) showed that purified phytase enzyme had high resistance to pepsin and retained at least 95% of its activity (0.53 $\mu\text{mol}/\text{min}$) at the varying levels of pepsin studied. The phytase of *E. coli* exhibited a comparable resistance to pepsin, whereas the phytases from *A. niger* and *P. lycii* lost 65% and 80% of their initial activity respectively at the highest level of pepsin. Trypsin treatment causes a considerable rise in phytase activity (Figure 1.1). Singh (2013) also reported enhanced phytase activity upon treatment with pepsin but observed a reduction in phytase when treated with trypsin.

Addition of gastric acid (GA) greatly reduced phytase activity (Figure 1.2). Phytase activity of 3.01 $\mu\text{mol}/\text{min}$ was observed in the medium without gastric acid (control). Inclusion of gastric acid reduced the enzyme to as low as 0.21 $\mu\text{mol}/\text{min}$ (about 97% reduction in phytase activity) ($P < 0.05$). The reduction in the phytase could be as a result of change in pH to a more acidic condition although only a few studies have examined the effect of gastric acid on phytase *in vitro*, Englmaierova *et al.* (2015) in an *in vivo* study reported that gastric acid addition decreased phytase activity which in turn decreased stomach pH. The report of Ekren and Metin (2013) revealed that there was an interaction of acid and phytase on average stomach pH. The addition of 3.0% gastric acid alone reduced stomach pH ($P < 0.001$) from 3.71 to 3.07. Phytase addition to the diet, with no added acid, increased stomach pH ($P < 0.001$) from 3.29 to 3.49. However, when phytase was added to the diet containing 3.0% gastric acid, stomach pH decreased to 3.02 ($P < 0.001$).

At a variety of substrate concentrations, phytase's kinetic parameters were measured. The value of K_M , which stands for the enzyme's affinity for a particular substrate, can change in response to environmental factors including pH, temperature, and ionic strength. K_M and V_{max} values of *Pseudomonas spp.* phytase were calculated from the Lineweaver–Burk plot as 0.0029 mM and 3.744 $\mu\text{mol}/\text{min}$ respectively (Figure 1.3). The Lineweaver–

Burk plot produced a linear regression equation:

$$\frac{1}{V} = 0.267 \left(\frac{1}{[S]} \right) + 0.267$$

With a correlation coefficient $R^2 = 0.97$. The K_M values of phytases are reported to range from 0.08 to 10 mM (Nayini and Markakis, 2016). The K_M values of phytases in the presence of sodium phytate substrate were reported to be 0.04, 0.295, 0.55, 0.03, and 2.19 mM in *K. pneumoniae* 9-3B, *A. ficuum* NTG-23, *Bacillus sp.*, *C. krusei*, and *B. subtilis* 168, respectively. (Ekren and Metin, 2013). This variation in the K_M value might be due to the difference in the microorganism.

Conclusion

In conclusion, the observed increase in phytase activity following protease treatment suggests that the enzyme retains activity in the presence of digestive proteases, although further studies are required to elucidate the underlying mechanism. Therefore, the study demonstrated that crude phytase from *Pseudomonas spp.* retained measurable activity in the presence of proteolytic enzymes. These findings suggest potential suitability of the enzyme for application in animal nutrition. However, further studies evaluating enzyme stability during feed processing and *in vivo* digestion are required.

Conflict of Interest

The authors declared no conflict of interest.

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REFERENCES

- Blaabjerg K, Jørgensen H, Tauson AH, Poulsen HD (2016) The presence of inositol phosphates in gastric pig digest is affected by time after feeding a non-fermented or fermented liquid wheat- and barley-based diet. *J. Anim. Sci.* **89**: 3153-3162.
- Boyce, A., and Walsh, G. (2006). Comparison of Selected Physicochemical Characteristics of Commercial Phytases Relevant to their Application in Phosphate Pollution Abatement. *Journal of Environmental Science & Health, Part A: Toxic/Hazardous Substances & Environmental Engineering* **41**, 789-798.

- Bulama B., Milala M. A., Abdurrahman A. A. and Esther I. (2017). Isolation and optimization of phytase from *Pseudomonas aeruginosa* isolated from poultry faeces. *Journal of Cell Biology and Biochemistry Research* Vol. 3(1), pp. 001-007
- Ekren, G.S.; Metin, K. (2013). Production, Purification and Characterization of Phytase from *Phytase Producing Fungus*, European Biotechnology Congress, Bratislava, Slovakia, May 16–18, *Current Opinion in Biotechnology*. **81**.
- Englmaierova, M., Skrivan, M., Skrivanova, E., Bubancova, I., Cermak, L., and Vlckova, J. (2015). Effects of a low-phosphorus diet and exogenous phytase on performance, egg quality, and bacterial colonisation and digestibility of minerals in the digestive tract of laying hens. *Czech J. Anim. Sci.* **60**, 542–549. doi:10.17221/8596-CJAS
- Greiner R, Gomes da Silva L, Couri S (2019). Purification and characterization of an extracellular phytase from *Aspergillus niger* 11T53A. *Brazilian J. Microbiol.* **40**: 795-807.
- Herrmann, K. R., Hofmann, I., Jungherz, D., Wittwer, M., Infanzón, B., Hamer, S. N. (2021). Generation of Phytase Chimeras with Low Sequence Identities and Improved thermal Stability. *J. Biotechnol.* **339**, 14–21. doi:10.1016/J.JBIOTECH.2021.07.005
- Herrmann, K. R., Ruff, A. J., and Schwaneberg, U. (2020). Phytase-based Phosphorus Recovery Process for 20 Distinct Press Cakes. *ACS Sustain. Chem. Eng.* **8**, 3913–3921. doi:10.1021/acssuschemeng.9b07433
- Herrmann, K. R., Ruff, A. J., Infanzón, B., and Schwaneberg, U. (2019). Engineered Phytases for Emerging Biotechnological Applications beyond Animal Feeding. *Appl. Microbiol. Biotechnol.* **103**, 6435–6448. doi:10.1007/s00253-019-09962-1
- Infanzón, B., Herrmann, K. R., Hofmann, I., Willbold, S., Ruff, A. J., and Schwaneberg, U. (2022). Phytase Blends for Enhanced Phosphorous Mobilization of Deoiled Seeds. *Enzyme Microb. Techn.* **153**, 109953. doi:10.1016/j.enzmictec.2021.109953.
- Kerovuo, S., da Costa Terzi, S., Saavedra Pinto, G. A., Pereira Freitas, S., and Augusto da Costa, A. C. (2020). Hydrolytic Enzyme Production in Solid-state Fermentation by *Pseudomonas syringae* 3T5B8. *Process Biochemistry* **36**: 255-261.
- Lazali, A., Acamovic, T., and Bedford, M. (2013). Phytic acid and Phytase: Implications for Protein Utilization by Poultry. *Poultry Science* **85**: 878-885.
- Maenz, S. K. (2019). Raw Material Selection and Medium Development for Industrial Fermentation Processes. In: Demain, A. L., Davies, J. E., *Manual of Industrial Microbiology and Biotechnology*. (2nd edition). ASM Press: Washington DC. pp: 213-220.
- Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, Van Loon AP (2017). Isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. *Microbiology* **143**: 245-252.
- Nayini, N.R.; Markakis, P. (2016). Phytic Acid: Chemistry and Applications; Graf, E.; Ed.; Pilatus Press: Minneapolis, MN; 101–118.
- Rousseau X, Létourneau-Montminy MP, Mème N, Magnin M, Nys Y, Narcy A (2012) Phosphorus utilization in finishing broiler chickens: effects of dietary calcium and microbial phytase. *Poult. Sci.* **91**: 28292837.
- Samson R.A, (2001). Introduction to Food Borne Microorganisms, *American Society of Microbiology*, America, pp 196-197.
- Sasirekha, B., Bedashree T and Champa KL (2012). Optimization and partial purification of extracellular phytase from *Pseudomonas aeruginosa* p6 *Euro. J. Exp. Bio.*, **2** (1):95- 104
- Selle, J. M., Roneker, K. R., Crowe, S. E., Pond, W. G., and Lei, X. G. (2012). Effectiveness of an Experimental Consensus Phytase in Improving Dietary Phytate-phosphorus Utilization by Weanling Pigs. *Journal of Animal Science* **81**: 2751-2757.

- Selvamohan, R., Alminger, M. L., and Carlsson, N. G. (2012). Stereospecificity of Myo-inositol Hexakisphosphate Dephosphorylation by a Phytate-degrading Enzyme of Baker's Yeast. *Journal of Agricultural Food Chemistry* **49**: 2228–2233.
- Singh B and Satyanarayana T (2008). Phytase Production by a Thermophilic Mould *Sporotrichum thermophile* in Solid State Fermentation and its Potential Applications. *Bioresource Technology* **99**: 2824–2830.
- Singh B, (2013). Improved production of protease-resistant phytase by *Aspergillus oryzae* and its applicability in the hydrolysis of insoluble phytates. *J Ind Microbiol Biotechnol* **40**: 891–899 DOI 10.1007/s10295-013-1277-3
- Tso, R., Lim, A. J., & Forde, C. G. (2020). A critical appraisal of the evidence supporting consumer motivations for alternative proteins. *Foods*, *10*(1), 24.