

OPTIMIZATION OF CULTURE CONDITIONS FOR PHYTASE PRODUCTION BY *ASPERGILLUS FLAVUS* PHY168 ISOLATED FROM SOIL

A. Ahmad^{1*}; A. A. Anjum²; M. Younus¹; S. Ahmed³; S. Ali⁵; S. Sana²; A. Asif²; N. Nazir⁴; A. Ashraf² and S.G. Mohyuddin⁶

¹Department of Pathobiology, University of Veterinary and Animal Sciences Lahore, Sub-campus Narowal, Pakistan

²Institute of Microbiology, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan

³Department of Basic Sciences, University of Veterinary and Animal Sciences Lahore, Sub-campus, 51600, Narowal, Pakistan

⁴Department of Zoology, Lahore College for Women University Lahore, Pakistan

⁵Research and Development Division, Foot and Mouth Disease Research Center, Zarrar Shaheed Road, Lahore Cantt. Pakistan 54810

⁶Department of Veterinary Medicine, Guangdong Ocean University, Zhanjiang, China

*Corresponding Author's Email: aliahmad@uvas.edu.pk

ABSTRACT: The present study deals with optimization of culture conditions for phytase production by indigenous strain of *Aspergillus flavus* PHY168 isolated from soil of livestock farms in Lahore district of Punjab, Pakistan. Best phytase producing isolate was selected based on size of zone of hydrolysis on plate of phytase screening medium agar and identified as *A. flavus* PHY168 by morphological methods. Culture conditions were optimized for maximum phytase production by *A. flavus* using one variable approach. Effect of temperature, pH, substrate type and concentration was studied using submerged fermentation. Results indicated that maximum phytase production was noted at temperature 35°C, pH 5 and 5% rice bran as substrate. Agricultural by-products such as rice bran can be used for cost effective phytase production under optimized conditions. Thus, indigenous *A. flavus* PHY168 can be used for large scale phytase production to meet its requirements in food and feed industries.

Key words: Phytase, *Aspergillus flavus*, Submerged fermentation, substrate.

(Received 10-07-2020

Accepted 10-09-2020)

INTRODUCTION

Phosphorus is an important mineral which is required for animal growth and production. The common feed ingredients in animal feeds are cereals and legumes. Phosphorus in feed ingredients is mainly stored in the form of compound called phytic acid (phytate) which represents 50-80 % of total phosphorus (Dersjant-Li *et al.*, 2015). Nonruminants such as poultry, swine and fish cannot secrete phytase to digest phytate. The phytate has anti-nutritional properties as it chelates important divalent cations / metals and proteins. The monogastric animals excrete phosphorus in feces which causes phosphorus pollution and eutrophication of water bodies in areas of intensive animal production (Azeem *et al.*, 2015; Vats *et al.*, 2005).

The phytate related issues are solved by phytase supplementation in of monogastric animal are poultry feed. The phytase attacks phytate to release inorganic phosphorus and lipids, magnesium, calcium and protein becomes available to the animal. It is naturally present in plants, animals and microorganisms (Humer *et al.*, 2014). Microbial Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is used in monogastric animal feeds to

hydrolyse phytate and release inorganic phosphate (Konietzky and Greiner, 2002). Microbial phytase supplementation in feed helps to reduce the phosphorus pollution and nutritional harms due to phytate (Kumar *et al.*, 2016). Thus, phytase improves the nutritional status of feed stuffs and digestibility of phytate for environment friendly poultry production (Nissar *et al.* 2017). Many species of fungi have potential of phytase production via fermentation of easily available cheap substrates (Arora *et al.*, 2017; Maurya *et al.*, 2017; Makolomakwa *et al.*, 2017; Shah *et al.*, 2017; karthik *et al.*, 2018; Dailin *et al.*, 2019). The present study was carried out to find and optimize culture conditions for maximum phytase production using native strain of *A. flavus*.

MATERIALS AND METHODS

Isolation and Identification of fungi: Phytase producing fungi were isolated after qualitative screening of soil samples on phytase screening medium (PSM) agar using the method described by Howson and Davis, (1983). The best phytase producer isolate was selected on the basis of size of zone of hydrolysis and identified as *A. flavus* PHY168 using morphological method described by

Tsuneo, (2010). It was maintained on slants of Sabouraud's Dextrose Agar at 4°C.

Optimization of culture conditions for phytase production: Culture conditions for maximum enzyme production by *A. flavus* PHY168 were optimized via submerged fermentation of phytase screening medium (PSM) broth using technique described by Singh and Satyanarayana, (2012). The PSM broth contained g/L: 15 g glucose, 5 g ammonium nitrate, 2 g calcium chloride, 0.5 g potassium chloride, 0.5 g magnesium sulphate, 0.01 g ferrous sulphate, 0.01 g manganese sulphate, 2 g sodium phytate and 1000 ml distilled water.

Inoculum preparation: Spores of pure *A. flavus* PHY168 were mixed in 10 ml sterilized normal saline and counted using neubar chamber. One ml spore suspension (1×10^7 spores) was used as inoculum to optimize culture conditions for phytase production.

Effect of temperature, pH and substrate on phytase production: To study effect of temperature on phytase production, 6 glass flasks (each having 50 ml sterilized PSM Broth with pH 5.5 inoculated with 1 mL standardized inoculum having 1×10^7 spores) were incubated at 6 temperatures ranging from 20°C to 45°C in shaking incubator for 5 days.

4 Glass flasks containing sterilized PSM broth with pH 3, 4, 5 and 6 respectively inoculated with one mL standardized inoculum (1×10^7 spores) and incubated in shaking incubator (150 rpm) for 5 days at temperature optimized in above mentioned step.

Effect of 1-5 % concentrations of each individual substrate such as rice bran, wheat bran and oat bran on phytase production was evaluated by incubation of inoculated sterilized PSM broth (at temperature and pH optimized in steps 1 & 2) in shaking incubator (150 rpm) for 5 days.

Filtration: Fungal growth of each parameter was filtered using Whatman filter paper No. 1. The filtrate was centrifuged to obtain supernatant.

Phytase Assay: The phytase assay was performed according to the colorimetric method of Fiske and Subarow (1925) to determine the amount of phytase. Both sample and control tubes were centrifuged at 4000 g for 10 minutes and kept at room temperature for 10 minutes. The spectrophotometer was blanked with the control (distilled H₂O) and respective absorbance (OD) values were taken at 660nm wavelength. The OD values were converted to phosphorus produced with the help of standard curve to determine phytase units. Standard curve was prepared using KH₂PO₄. One Phytase unit (U) was defined as the amount of enzyme that released 1 umol of inorganic phosphorous per minute per mL of supernatant under the assay conditions.

Determination of Biomass (g/L): The Fungal biomass for each parameter was determined after drying the filter paper containing filtered mycelium at 60°C in hot air oven for 6 hours and its dry weight expressed as g / L medium as described by Lata *et al.* (2013).

Statistical Analysis: Mean values of phytase and fungal biomass were compared by One-way Analysis of Variance (ANOVA) and Post- Hoc Multiple comparison test (Duncan) using SPSS (version 20.0). Significance was declared at $p < 0.05$.

RESULTS AND DISCUSSION

In the present study, efforts were made to optimize physical and chemical conditions to increase enzyme production by *Aspergillus flavus* PHY168 through submerged fermentation. The effect of incubation temperature ranging from 20°C - 45°C on phytase production and fungal growth was investigated using one variable approach. Present results revealed that *A. flavus* PHY168 produced maximum phytase (3.54 U) and biomass (13.27 g/L) in PSM broth at 35°C (Table 1). Like our results, Jafari-Tapeh *et al.*, 2012 described maximum phytase production at optimum temperature of 35°C. It was noted that enzyme yield is growth associated and affected significantly by performing fermentation above or below the optimum temperature of incubation. Various culture conditions affect production of phytase by fungi (Qasim *et al.*, 2017; Neira-Vielma *et al.*, 2018). Microbial species produce phytase within a broad range of optimal physical conditions (pH and temperature). For digestion of feed in simple stomach animals, enzyme must be thermo-tolerant so that it can withstand high temperature during feed production (Mittal *et al.*, 2012).

A. flavus PHY168 produced maximum phytase (3.61 U) and biomass (14.28 g/L) at optimum pH of 5 (Table 2). The PSM broth with pH ranging from 3-6 was used to produce enzyme and fungal biomass under submerged fermentation by incubation at optimized temperature. Our results indicated *A. flavus* PHY168 showed maximum phytase production at optimum PH of 5. Similar to our results, previous studies showed maximum phytase production at pH 5 (Singh and Satyanarayana., 2008; Sandhya *et al.*, 2015 ; Qasim *et al.*, 2017). The production of phytase is mostly reported in acidic to neutral pH range. Optimum pH ranges from 3.0 - 5.5 and 7.0-8.0 for acidic phytase & alkaline phytase respectively (Yin *et al.*, 2007; Vijayaraghavan *et al.*, 2013).

Results for phytase production and fungal biomass by growth of *A. flavus* PHY168 in PSM broth supplemented with 1 %, 2 %, 3 %, 4 % and 5 % concentration of rice bran, wheat bran and oat bran are presented in Table 3. *A. flavus* PHY168 produced maximum phytase (7.14 U) and biomass (28.49 g/L)

using optimum concentration of 5% rice bran. It produced maximum phytase (5.76 U) and highest biomass (22.69 g/L) with 4 % wheat bran in PSM broth. Our results revealed maximum amount of phytase (5.44 U) and biomass (21.68 g/L) using 5 % oat bran in PSM. In the present study, 3 substrates were tested for production of phytase using submerged fermentation. Order of substrate choice for highest phytase production is rice bran > wheat bran > oat bran. It is concluded that fermentation medium containing 5 % rice bran showed maximum enzyme yield and fungal biomass growth as compared to wheat bran and oat bran. Present results are in accordance with previous studies showing maximum enzyme production using rice bran as substrate (Bhavsar *et al.*, 2008; Suresh and Radha, 2015; Gunashree and Venkateswaran, 2008).

A variety of agricultural by-products and residues as substrate for enzyme production by various species of fungi were used in several studies (Huang *et al.*, 2018; Shahryari *et al.*, 2018; Pires *et al.*, 2019; Jatuwong *et al.*, 2020). These are available in large quantities at low price in Pakistan and can be used to produce enzymes on industrial level via fermentation. Thus our study suggests use of rice bran as substrate to produce phytase for applications in food and feed industries.

It was found that selected fungal isolate produced maximum enzyme and biomass at optimized physical and chemical parameters. Several studies have been conducted to optimize culture condition for phytase production by different species of fungi through fermentation (Lata *et al.*, 2013; Sabu *et al.*, 2002; Krishna *et al.*, 2001).

Table 1: Effect of temperature on phytase production.

Temperature	20°C	25°C	30°C	35°C	40°C	45°C
Enzyme	0.86 ± 0.04 ^f	1.54 ± 0.05 ^e	2.25 ± 0.06 ^c	3.54 ± 0.03 ^a	2.62 ± 0.05 ^b	1.93 ± 0.04 ^d
Biomass	3.44 ± 0.11 ^f	4.52 ± 0.05 ^e	8.44 ± 0.08 ^c	13.27 ± 0.06 ^a	9.78 ± 0.07 ^b	7.25 ± 0.04 ^d

Means with different superscripts indicate that values are statistically significant (P<0.05).

Table 2: Effect of pH on phytase production.

pH	3	4	5	6
Enzyme	1.62 ± 0.02 ^d	2.41 ± 0.03 ^b	3.61 ± 0.03 ^a	2.33 ± 0.04 ^c
Biomass	6.49 ± 0.06 ^d	9.69 ± 0.07 ^b	14.28 ± 0.11 ^a	9.31 ± 0.08 ^c

Means with different superscripts indicate that values differ significantly (P<0.05)

Table 3: Effect of Substrates on phytase production and biomass (g/L).

Substrate	Concentration	1%	2%	3%	4%	5%
Rice Bran	Enzyme	4.38 ± 0.05 ^e	5.84 ± 0.06 ^d	6.81 ± 0.05 ^c	6.93 ± 0.04 ^b	7.15 ± 0.04 ^a
	Biomass	17.46 ± 0.10 ^e	23.43 ± 0.07 ^d	27.32 ± 0.08 ^c	27.70 ± 0.09 ^b	28.49 ± 0.06 ^a
Wheat Bran	Enzyme	3.51 ± 0.02 ^e	4.63 ± 0.02 ^d	5.26 ± 0.04 ^b	5.76 ± 0.03 ^a	4.95 ± 0.03 ^c
	Biomass	14.06 ± 0.10 ^e	18.51 ± 0.04 ^d	21.08 ± 0.05 ^b	22.69 ± 0.11 ^a	19.75 ± 0.09 ^c
Oat Bran	Enzyme	2.96 ± 0.03 ^e	3.41 ± 0.02 ^d	3.85 ± 0.03 ^c	4.77 ± 0.06 ^b	5.44 ± 0.04 ^a
	Biomass	11.82 ± 0.05 ^e	13.55 ± 0.07 ^d	14.34 ± 0.06 ^c	19.10 ± 0.06 ^b	21.68 ± 0.09 ^a

Means with different superscripts indicate that values differ significantly (P<0.05).

Conclusion: Present study suggests that indigenous *A. flavus* PHY168 strain can be cultured at temperature 35°C, 5 pH 5 and 5 % rice bran for phytase production in less cost on industrial scale.

REFERENCES

- Arora, S., M. Dubey, P. Singh, R. Rani and S. Ghosh (2017). Effect of mixing events on the production of a thermo-tolerant and acid-stable phytase in a novel solid-state fermentation bioreactor. *Pro. Biochem.* 61, pp.12-23.
- Azeem, M., A. Riaz, A.N. Chaudhary, R. Hayat, Q. Hussain, M.I. Tahir and M. Imran (2015). Microbial phytase activity and their role in organic P mineralization. *Arch. Agro. Soil Sci.*, 61, 751–766.
- Bhavsar, K., P. Shah, S.K. Soni and J.M. Khire (2008). Influence of pretreatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. *Afr. J. Biotech.* 7(8).
- Dailin, D.J., S.Z. Hanapi, E.A. Elsayed, D. Sukmawati, N.I.W. Azelee, J. Eyahmalay, V. Siwapiragam and H. El Enshasy (2019). Fungal phytases:

- biotechnological applications in food and feed industries. Rec. Adv. W. Biotechnol. Fun. (pp. 65-99). Springer, Cham.
- Dersjant-Li, Y., A. Awati, H. Schulze and G. Partridge (2015). Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. J. Sci. Food. Agric. 95(5), 878-896.
- Fiske, C.H., Y. Subbarow (1925). The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.
- Gull, I., A. Hameed, M.S. Aslam and M.A. Athar (2013). Optimization of phytase production in solid state fermentation by different fungi. Afri. J. Micr. Res. 7(46):5207-5212.
- Gunashree, B.S. and G. Venkateswaran (2008). Effect of different cultural conditions for phytase production by *Aspergillus niger* CFR 335 in submerged and solid-state fermentations. J. Ind. Microbiol Biotech. 35(12):1587-1596.
- Huang, S. J., C.H. Chen and S.Y. Tsai (2018). Phytase production by *Grifola Frondosa* and its application in inositol-enriched solid-state fermentation brown rice. Int. J. Food Eng. 4, 263-267
- Howson, S.J. and Davis, R.P 1983. Production of phytate-hydrolysing enzyme by some fungi. *Enzyme and Microbial Technology*. 5(5), pp.377-382.
- Humer, E., C. Schwarz and K. Schedle (2014). Phytate in pig and poultry nutrition. J. Anim. Physiol. Anim. Nutr 99(4):605-625
- Jafari-Tapeh, H., Z. Hamidi-Esfahani and M. H. Azizi (2012). Culture condition improvement for phytase production in solid state fermentation by *Aspergillus ficuum* using statistical method. ISRN Chem. Eng. 2012, 1-5.
- Jatuwong, K., N. Suwannarach, J. Kumla, W. Penkhru, P.Kakumyan, P. and S. Lumyong (2020). Bioprocess for Production, Characteristics, and Biotechnological Applications of Fungal Phytases. Front. Micr. 11, p.188.
- Karthik, N., P. Binod and A. Pandey, (2018). SSF production, purification and characterization of chitin deacetylase from *Aspergillus flavus*. Biocatal. Biotransfor. 36, 296-306
- Konietzny, U. and R. Greiner (2004). Bacterial phytase: potential application, *in vivo* function and regulation of its synthesis. Braz. J. Micr. 35(1-2):12-18
- Konietzny, U., and R. Greiner (2002). Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. J. F. Sci. Tech. 37(7):791-812.
- Krishna, C. and S.E. Nokes (2001). Influence of inoculum size on phytase production and growth in solid-state fermentation by *Aspergillus niger*. Trans. ASAE. 44(4):1031.
- Kumar, A., A. Chanderman, M. Makolomakwa, K. Perumal and S. Singh, (2016). Microbial production of phytases for combating environmental phosphate pollution and other diverse applications. Crit. Rev. Environ. Sci. Techn. 46(6), pp.556-591.
- Lata, S., S. Rastogi, A. Kapoor and M. Imran (2013). Opimization of culture conditions for the production of phytase from *Aspergillus heteromorphus* MTCC 10685. Int. J. Adv. Biotech. 4(2):224-235.
- Makolomakwa M, A.K. Puri, P. Kugen, S. Singh (2017) Thermo-acid-stable phytase-mediated enhancement of bioethanol production using *Colocasia esculenta*. Bioresour. Technol. 235:396-404
- Maurya A. K, D. Parashar, T. Satyanarayana (2017) Bioprocess for the production of recombinant HAP phytase of the thermophilic mold *Sporotrichum thermophile* and its structural and biochemical characteristics. Int. J. Biol. Macromol. 94:36-44.
- Mittal, A., G. Singh, V. Goyal, A. Yadav and N.K. Aggarwal (2012). Isolation and biochemical characterization of acido-thermophilic phytase producer yeast strain for potential application in poultry feed. Adv. in App. Res. 4(1):26-34.
- Neira-Vielma, A. A., C.N. Aguilar, A. Ilyina, J.C. Contreras-Esquivel, M.G. Carneiroda-Cunha, G. Michelena-Álvarez, (2018). Optimized production of phytase by solid-state fermentation using triticale as substrate and source of inducer. Afr. J. Biotech. 17, 81-90.
- Nissar, J., T. Ahad, H.R. Naik, S.Z. Hussain (2017). A review phytic acid: As antinutrient or nutraceutical. J. Pharmacog. Phytochem. 6(6):1554-60.
- Pires, E.B.E., A.J. de Freitas, F.F. e Souza, R.L. Salgado, V.M. Guimarães, F.A. Pereira and M.R. Eller (2019). Production of Fungal phytases from Agroindustrial Byproducts for pig Diets. Scient. Rep. 9(1), pp.1-9.
- Qasim, S. S., K.A. Shakir, A. B. Al-Shaibani, and M.K. Walsh (2017). Optimization of culture conditions to produce phytase from *Aspergillus tubingensis* SKA. F. Sci. Nutr. 8, 733-745.
- Roopesh, K., S. Ramachandran, K.M. Nampoothiri, G. Szakacs and A. Pandey (2006). Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*. Biores. Tech. 97(3):506-511.
- Sabu, A., S. Sarita, A. Pandey, B. Bogar, G. Szakacs and C.R. Soccol (2002). Solid-state fermentation for

- production of phytase by *Rhizopus oligosporus*. App. Bioch. Biotech. 102(1-6):251-260.
- Sandhya, A., A. Sridevi, D. Suvarnalatha and G. Narasimha (2015). Production and optimization of phytase by *Aspergillus niger*. Pharm. Lett. 7, 148–153.
- Shah, P. C., V. R. Kumar, S. G. Dastager, and J.M. Khire (2017). Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. AMB Exp. 7, 1–11.
- Shahryari, Z., M. H. Fazelipour, P. Setoodeh, R.B. Nair, M.J. Taherzadeh and Y. Ghasemi, 2018. Utilization of wheat straw for fungal phytase production. Int. J. Recy. Org. W. Agri. 7(4), pp.345-355.
- Singh, B., and T. Satyanarayana (2008). Improved phytase production by a thermophilic mould *Sporotrichum thermophile* in submerged fermentation due to statistical optimization. Bioresour. Technol. 99, 824–830
- Suresh, S. and K.V. Radha (2015). Effect of a mixed substrate on phytase production by *Rhizopus oligosporus* MTCC 556 using solid state fermentation and determination of dephytinization activities in food grains. F. Sci. Biotech. 24(2):551-559.
- Tsuneo, W (2010). Pictorial atlas of soil and seed fungi: Morphologies of cultural fungi and Key to Species. Third editions, CRC press 2010.
- Vats, P., M.S. Bhattacharyya and U.C. Banerjee (2005). Use of phytases (myo-inositolhexakisphosphate phosphohydrolases) for combatting environmental pollution: A biological approach. Crit. Rev. Envir. Sci. Tech. 35, 469–486.
- Vijayaraghavan, P., P.R. Primiya, S.G.P. Vincent (2013). Thermostable alkaline phytase from *Alcaligenes* sp. in improving bioavailability of phosphorus in animal feed. In vitro analysis. ISRN Biotechnol. 1-6.
- Yin, Q. Q., Q.H. Zheng, X.T. Kang (2007). Biochemical characteristics of phytases from fungi and the transformed microorganism. Anim. F. Sci Technol. 132, 341-350.