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Research Article

Optimization of Culture Conditions for Phytase Production by *Aspergillus flavus* PHY168 isolated from soil

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ABSTRACT

A large number of microbes have potential to produce inorganic phosphorus releasing enzyme Phytase. It has commercial uses in food and feed industries. The present study was conducted for characterization of a novel strain of *Aspergillus flavus* PHY168 isolated from soil of livestock farms in Lahore district of Punjab. The inoculated plates of phytate specific medium with spores of fungal isolates were incubated for 96 hours and observed for zone of hydrolysis. Selected isolate was identified by morphological method. Physical and chemical conditions were optimized for optimal production of phytase by *Aspergillus flavus* using the one variable technique. Four different factors such as temperature, pH, substrate type and concentration were studied. Best phytase producing isolate was selected based on size of zone of hydrolysis on PSM plate and identified as *Aspergillus flavus* PHY168 by using morphological and molecular methods. Optimal temperature and pH were found to be 35^{0}C and 5 respectively. Maximum phytase production was observed as 3.53 µm/mL at temperature 35^{0}C, 3.60 µm/mL at pH 5 and 7.13 µm/mL at 5% rice bran concentration. Agricultural bye-products such as rice bran can be used for cost effective phytase production using optimized culture conditions. Thus, indigenous *A. flavus* PHY168 can be used for large scale phytase production to meet its need in food and feed industries.

Key words: *Aspergillus flavus*, Phytase, Characterization, Optimization, Submerged fermentation.

Introduction:

Phosphorus is one of the most important nutrients required for chicken growth. Cereal grains, legumes and oil seeds are the principal components of feed and contain phosphorus in organic form (phytate). Almost 75 percent phosphorus is present in the form of phytic acid (phytate) in oil seeds and grains, (Ahmad et al. 2000; Maenz, 2001). Monogastric animals like poultry do not produce phytase to digest phytate. As a result, the phytate acts as anti nutritional agent and undigested phosphorus passes out as

excreta into the environment where it causes phosphorus pollution in areas of intensive livestock production. When phosphorus goes into aquatic environment, it disturbs water quality which is known as eutrophication. Thus Phosphorus is used inadequately by poultry which results in environmental complications and economical loss (Cromwell and Coffey, 1991).

Supplementing poultry feed with phytase is an effective way to digest the phytate to improve the availability of phosphate, protein and divalent cations (Nelson et al. 1971). Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) of fungal origin is added in the feeds of monogastric animals for hydrolysis of phytic acid (*myo*inositol hexakisdihydrogen phosphate) to the Mono-, Di-, Tri-, Tetra-, and Pentaphosphates of *myo*-inositol and inorganic phosphate (Simell et al. 1989). Phytase has both microbial and nonmicrobial source. Its non microbial source includes plants and animals where it is naturally present. Microbial sources include a large number of bacteria and fungi which produce phytase through fermentation using a great variety of substrates.

Phytase is the major feed component of simple stomach animals to increase the nutritive quality of plants by releasing phosphate (Konietzny and Greiner 2004; Farouk et al. 2015). The quality of feed from plant based sources can be increased by adding phytase which increases the digestibility and accessibility of proteins by hydrolyzing phytate (Farouk et al. 2012). Phytase produced by micro-organisms is utilized in feed supplements to handle the ecological and nutritional harms due to phytate (Gonita-Mishra et al. 2013). Phytase not only provides free phosphorus but also plays role in the availability of lipids, magnesium, calcium and protein. By liberating inorganic phosphorus from bounded source, phytase provides phosphorus in higher quantity to the poultry birds for bone growth and save the ecological system from pollution. Thus microbial phytase can be used to exploit the inherent nutritional potential of feed stuffs for more economic and environment friendly poultry production (Singh et al 2014). Therefore, for the reduction of production costs, it is desirable to isolate phytase producing fungi from indigenous soil that have the properties of enzyme such as pH and temperature (Shieh and Ware, 1968; Mitchel et al 1997 and Lee et al., 2005). Keeping in view the increasing commercial importance of phytase for food and feed industries, present study was conducted to optimize physical and chemicals conditions for phytase production.

Materials and methods:

Pure fungal isolates recovered from soil samples of livestock farms were qualitatively screened on phytase screening medium (PSM) agar for the presence of phytase producing fungi by using the method described by Howson and Davis, 1983. Spores from each pure fungal isolate were inoculated on PSM agar plate. The plates were incubated at 25°C for 48 hours to 96 hours. The colonies surrounded by zone of hydrolysis on PSM plates were considered phytase positive and purified cultures stored on slants at 4^0C.

Identification of Fungi:

The best phytase producing isolate was selected on the basis of size of zone of hydrolysis and identified by morphological method using macroscopic and microscopic characters as described by Tsuneo, (2010).

Optimization of culture conditions for phytase production:

Both physical and chemical conditions were optimized to increase enzyme production by selected twelve isolates under submerged fermentation using one variable approach described by Singh and Satyanarayana, 2012. The physical conditions included temperature and pH while chemical conditions included substrate type and concentration.

Inoculum preparation:

The inoculum was prepared by counting fungal spores with the help of neubar chamber using the method described by Morris and Nicholls (1978). Normal saline in screw capped glass test tubes (10mL/tube) was sterilized by autoclaving at 121C for 15 min under 15lb / $inch^2$. After autoclaving it was allowed to cool and a loop full of spores was shifted to tube within six inches of flame. A uniform spore suspension was prepared by mixing the spores in normal saline with gentle shaking. For counting 10 uL of spore suspension was transferred on neubar counting chamber of haemocytometer with the help of micropipette. It was covered with cover slip. Spore counting was done at 100X magnification. The spores were counted in five boxes and average value in one box was calculated by following formula:

1. Number of spores / box= Total spores in 5 boxes / 5
2. The average number of spores in one box was divided by 0.004 (volume of one box) to get spores in 1 uL. By unit method spores in 10uL and in one mL were calculated. Spore inoculum 1 x 10^7 spores / mL was used for optimization of conditions for enzyme productions.

Effect of temperature on phytase production:

PSM broth was prepared by mixing its ingredients in the 5 L Erlenmeyer flask. Litre composition of PSM was: 15 g glucose, 5 g NH_4NO_3, 2 g $CaCl_2$, 0.5 KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.01 g MnSO4.H2O, 2 g sodium phytate. pH was adjusted to 5.5 with the help of 0.1N NaOH and 0.1 N HCl using pH meter. Seventy two clean glass flasks of 500 ml capacity were taken and 50mL PSM broth was filled in each flask. All flasks were sterilized by autoclaving at 121^0C for 15 minutes. After sterilization, gentamycine (50ug/mL) was added to stop the bacterial growth. One mL standardized inoculum ($1x10^7$ spores / 50 ml medium) was transferred to each flask aseptically. Six flasks inoculated with each isolate were incubated in shaking incubators (150 rpm) at 20^0C, 25^0C, 30^0C, 35^0C, 40^0C and 45^0C for 5 days.

Filtration:

After 5 days of incubation, filtration was done by Whatman filter paper No. 1. The filter paper was folded and kept inside a clean glass funnel. Filtration apparatus was set using glass beaker and glass funnel hanging over it with the help of support of tripod stand. Filter paper was placed in cone of funnel with three folds on one side and one on other side. Poured all the material including fungal growth and medium onto filter paper and let the process to run. The filtrate was used for determination of phytase quantity by performing Phytase assay. The filter paper

containing filtered mycelium was dried at 60^0C in hot air oven for 6-24 hours to determine the fungal biomass and expressed as g dry weight/L medium as described by Lata et al. (2013).

Phytase assay:

For quantification of enzyme production, phytase assay was performed by following method as described by Fiske and Subarrow (1925). Both sample and control tubes were centrifuged at 4000 g for 10 minutes and kept at room temperature for10 minutes. A spectrophotometer was switched on 15 minutes earlier before use. It was blanked with the control (distilled water) and OD values were taken at 660nm wavelength by filling cuvette with 2 mL reaction mixture. All reactions were carried out in triplicate. The OD values were converted to phosphorus produced with the help of standard curve for determination of phytase units. Standard curve was prepared using KH_2PO_4 with the help of known concentration and by taking their respective absorbance (OD) values. One Phytase unit (U) was defined as the amount of enzyme that released 1 umol of inorganic phosphorous per minute per mL of culture filtrate under the assay conditions (pH 5.5, 37 °C).

Effect of pH on phytase production:

PSM broth one litre was prepared by mixing its ingredients in each of 4 properly labeled glass flasks. pH of respective flasks was adjusted to 3, 4, 5 and 6 respectively. Each glass flask was filled with 50mL PSM broth and sterilized by autoclaving at 121^0C. After sterilization gentamycine (50ug/mL) was added to stop the bacterial growth. One mL from standardized inoculums (1 x 10^7 spores) was transferred aseptically to each properly labeled flask. All the flasks were incubated in shaking incubator (150 rpm) for 5 days at temperature optimized for respective isolate. After incubation of 5 days, filtration of broth was done using Whatman No. 1 filter paper. The filtrates were centrifuged and supernatant was used as source of enzyme for phytase assay. Amount of inorganic phosphorus released was determined with the help of standard curve and enzyme activity was determined for each isolate under different pH conditions. Filter paper containing filtered mycelium was dried at 60^0C in hot air oven for determination of fungal biomass of each isolate at given pH.

Effect of substrate concentration on phytase production:

Effect of various concentrations (1-5 %) of three different substrates (Rice bran, wheat bran and oat bran) on phytase production was evaluated by incubation of PSM broth (optimized pH) at optimized temperature for each isolate. PSM broth (pH 5) of 5 different concentrations (1-5%) for each substrate was prepared in 5 separate one litre glass flasks having 1%, 2% , 3% , 4% , 5% concentration respectively. Each glass flask was filled with 50mL PSM broth and sterilized by autoclaving at 121^0C. After sterilization, gentamycine (50ug/mL) was added to stop the bacterial growth. One mL from standardized inoculums (1 x 10^7 spores) was transferred aseptically to each properly labeled flask. All the flasks were incubated in shaking incubator (150 rpm) for 5 days at temperature optimized for respective isolate. After incubation of 5 days, filtration of broth was done using Whatman No. 1 filter paper. The filtrates were centrifuged and supernatant was used as enzyme source for phytase assay. Amount of inorganic phosphorus released was determined with the help of standard curve and enzyme activity was determined for each isolate under different substrate

concentrations. Filter paper containing filtered mycelium was dried at 60^0C in hot air oven for determination of fungal biomass of each isolate at given concentration of each substrate.

Statistical Analysis :

Studies were performed in triplicates (n=3) and mean value of enzyme production was calculated. One-way Analysis of Variance (ANOVA) and Post- Hoc Multiple comparison test (Duncan) was performed to compare the differences between means using SPSS (version 20.0) Significance was declared at $p < 0.05$.

Results:

Physical and chemical conditions were optimized to increase the enzyme production by phytase producing strains of *Aspergillus niger* PHY82.

Phytase production at different temperatures is given in Table 1. Phytase activity (U/mL) of cell free supernatant by performing phytase assay, was calculated from a standard curve. *A. flavus* PHY168 produced maximum quantity of phytase (3.53 U) in PSM broth at optimum temperature of 35^0C. Present results revealed that *A. flavus* PHY168 had maximum biomass value (13.28 g/L) at 35^0C.

Phytase production in PSM broth with pH ranging from 3-6 was evaluated by measuring the enzyme units per mL. Results showed that all *A. flavus* PHY168 produced maximum phytase (3.6 U) at optimum pH of 5 (Table 2). Fungal biomass production (g/L) for selected fungi was calculated at different pH (Table 2). *A. flavus* PHY168 produced maximum biomass (14.27 g/L) at optimum pH 5.

Effect of Chemical Conditions on Phytase Production:

Optimal chemical conditions for enzyme production were evaluated by culturing fungal isolates in PSM broth supplemented with 5 different concentrations (1 %, 2 %, 3 %, 4 % and 5 %) of each substrate (rice bran, wheat bran and oat bran).

Effect of rice bran on phytase production:

Effect of rice bran was evaluated by measuring the enzyme units per mL. Enzyme production in PSM broth supplemented with rice bran concentration ranging from 1-5 percent, under submerged fermentation is shown in table 3. Present results showed that *A. flavus* PHY168 produced maximum phytase (7.13 U) and biomass (28.47 g/L) using optimum concentration of 5% rice bran.

Effect of wheat bran on phytase production:

Effect of wheat bran (1-5 percent) on phytase production under submerged fermentation is shown in table 4. Present results revealed that *A. flavus* PHY168 produced maximum phytase (5.75 U) and highest biomass (22.68 g/L) with 4 % wheat bran in PSM broth

Effect of oat bran on phytase production:

Phytase production in PSM broth supplemented with oat bran concentration ranging from 1-5 percent, under submerged fermentation is shown in table 5. Present results showed that isolate *A. flavus* PHY168 produced maximum phytase (5.42 U) and biomass (21.67 g/L) in PSM broth supplemented with 5 % oat bran.

Table 1. Effect of temperature on phytase production

Temperature	20°C	25°C	30°C	35°C	40°C	45°C
Enzyme	0.87 ±0.04^{f}	1.53 ±0.05^{e}	2.24 ±0.06^{c}	3.53 ±0.03^{a}	2.61 ±0.05^{b}	1.92 ±0.04^{d}
Biomass	3.43±0.11^{f}	4.51±0.05^{e}	8.43±0.08^{c}	13.28±0.06^{a}	9.79±0.07^{b}	7.26±0.04^{d}

Data was analyzed by mean±. Means in same row 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	1.61±0.02^{d}	2.42 ±0.03^{b}	3.60 ±0.03^{a}	2.32 ±0.04^{c}
Biomass	6.48±0.06^{d}	9.70±0.07^{b}	14.27±0.11^{a}	9.30±0.08^{c}

Data was analyzed by mean±. Means in same row with different superscripts indicate that values are statistically significant (P<0.05)

Table 3. Effect of rice bran on phytase production and biomass (g/L)

Concentration	1%	2%	3%	4%	5%
Enzyme	4.37±0.05^{e}	5.83 ±0.06^{d}	6.80±0.05^{c}	6.92±0.04^{b}	7.13±0.04^{a}
Biomass	17.45 ±0.10^{e}	23.42 ±0.07^{d}	27.31 ±0.08^{c}	27.69 ±0.09^{b}	28.47 ±0.06^{a}

Data was analyzed by mean±. Means in same row with different superscripts indicate that values are statistically significant (P<0.05).

Table 4. Effect of Wheat bran on phytase production and biomass (g/L)

Concentration	1%	2%	3%	4%	5%
Enzyme	3.50±0.02^{e}	4.62 ±0.02^{d}	5.25 ±0.04^{b}	5.75 ±0.03^{a}	4.94 ±0.03^{c}
Biomass	14.05 ±0.10^{e}	18.50±0.04^{d}	21.07±0.05^{b}	22.68±0.11^{a}	19.74±0.09^{c}

Data was analyzed by mean±. Means in same row with different superscripts indicate that values are statistically significant (P<0.05).

Table 5. Effect of oat bran on phytase production and biomass (g/L)

Concentration	1%	2%	3%	4%	5%
Enzyme	2.95±0.03^{e}	3.40 ±0.02^{d}	3.84 ±0.03^{c}	4.76 ±0.06^{b}	5.42 ±0.04^{a}
Biomass	11.80±0.05^{e}	13.54 ±0.07^{d}	14.33 ±0.06^{c}	19.08±0.06^{b}	21.67±0.09^{a}

Data was analysed by mean±. Means in same row with different superscripts indicate that values are statistically significant ($P<0.05$).

Discussion:

Enzyme production and fungal growth under submerged fermentation was studied by incubation of inoculated standard PSM broth at six different temperatures for 5 days. The maximum phytase production as well as growth was observed at optimum temperature of 35^0C.

Effect of temperature on enzyme production and fungal growth under submerged fermentation was studied by incubation of inoculated standard PSM broth at six different temperatures for 5 days. It was observed that fungal isolates showed maximum phytase production as well as growth at their optimum temperature. The results of our study clearly established the fact that phytase production is growth associated as well and fermentation performed above or below the optimum growth temperature significantly affects the enzyme yield. The effect of temperature varying between 20^0C and 45^0C on phytase production and growth was determined. The fermentation temperature for optimum production of phytase is mostly reported as 30^0C by many researchers. For instance Tahir et al. (2010) reported maximum phyatse production from *Aspergillus niger* at 30^0C. Similarly, Lata et al. (2013) found that *Aspergillus heteromorphus* secreted maximum phytase at 30^0C. Similar reports were made by Soni and khire. (2007) and Vats and Benerjee et al. (2002).

A broad range of optimal pH and temperature values for phytase activity has been reported in the literature across microbial species. Temperature affects various metabolic processes such as protein denaturation, enzymatic inhibition, promotion or inhibition of a particular metabolite, cell death etc. For food digestion in stomach of monogastric animals, enzyme has to be thermo-tolerant so that that can withstand high temperature during feed production (pelleting).

Using optimized temperature, PSM broth with pH ranging from 3-6 was used for enzyme production and fungal biomass under submerged fermentation. *Aspergillus flavus* PHY168 showed maximum phytase production at optimum PH of 5. There is no report of phytase production at alkaline pH. Similar results were reported by Singh and Satyanarayana (2012) who stated that *Sporotrichum thermophile* produced maximum phytase at pH 5. Phytase production mostly reported in acidic to neutral pH range. This is in agreement with the report of Gull et al. (2013) which indicated phytase production by *Aspergillus flavus* at the same pH. These findings corroborate several investigations that reported acidic to neutral pH of fermentation medium favour the optimal phytase production (Howson and Davis, 1983; Shimizu, 1993; Sano et al., 1999; Andlid et al., 2004; Gulati et al., 2007; Singh and Satyanarayana, 2012; Selvamohan et al., 2012). Similarly, Gunashree and Vankateswaran (2008) reported phytase production under either submerged or solid state fermentation at pH 4.5 only. Our finding is also similar to those of Gaind and Singh (2015) who studied production, purification and characterization of neutral phytase from non-toxigenic (aflatoxin negative) thermotolerant *Aspergillus flavus* ITCC 6720 and reported maximum phytase production at optimum pH of 6. Our results showed that majority of selected fungal

strains produced maximum phytase at optimum pH of 5 which is close to the pH of poultry digestive tract where phytase acts on phytate. The enzyme with optimum pH near neutral are preferred as feed additive for poultry since this optimum pH is close to the physiological pH of poultry crop and aquaculture species (Jorquera et al., 2008).

Carbohydrates are most commonly used as substrates for enzyme production by microorganisms under fermentation. These include agricultural by-products, such as wheat bran, rice husk, rice bran and oat bran. These substrates serve not only as the source of carbon but also provide organic nitrogen (amino acid), vitamins and minerals. In the present study, rice bran, wheat bran and oat bran (1-5 percent) were used for production of phytase and fungal biomass under submerged fermented

Effect of different concentrations (1, 2, 3, 4 and 5 %) of each substrate on phytase production under submerged fermentation by selected isolate of *A. flavus* was studied at optimized temperature and pH. By culturing fungi on rice bran, *A. flavus* (PHY168) produced maximum phytase using optimum concentration of 5% rice bran. All concentrations of rice bran supported microbial growth and enzyme production. it showed maximum enzyme production using optimum concentration of 4% wheat bran and 5% oat bran. Order of substrate choice for highest phytase production is rice bran > wheat bran > oat bran. It is concluded that rice bran supported maximum fungal growth and higher enzyme production as compared to wheat bran and oat bran. Thus rice bran can be used to produce phytase for poultry feed industry.

Present results are in good accordance with previous studies showing maximum enzyme production using rice bran as substrate. For instance in a similar study, Bhavsar et al. (2008) evaluated influence of agriculture residues (rice bran, de-oiled rice bran, wheat bran, peanut cake, chickpea, maize and coconut cake) on phytase production under submerged fermentation by *Aspergillus niger* NCIM 563 and reported higher enzyme activity using rice bran as comared to wheat bran and other substrates. Similarly Suresh and Radha (2015); Gunashree and Venkateswaran (2008) also reported rice bran as good substrates for phytase production by *Rhizopus oligosporus*, *Aspergillus ficcum* and *Aspergillus niger*. Based on this the rice bran has been used as an alternative substrate for phytase production in subsequent studies. Contrary to our results, Gaind and Singh (2015) used different agro-industrial residues including wheat bran, rice bran, wheat & rice bran mixture, mustard cake, fruit pulp, oat meal and soybean meal and reported that *A. flavus* ITCC 6720 produced maximum phytase by utilization of mustard cake. Our results are not in agreement with those of Gull et al. (2013) who studied fungal isolates (*A. niger*, *A. flavus*, *A. versicolor*, *A. nidulans*, *C. cladosporioides*, *T. reesei*, and *T. viride*) to explore their potential of phytase production using wheat bran, lentil, oat, corn and bagasse and reported maximum phytase production by *Aspergillus flavus* using wheat bran as best substrate. Contrary to our results, Singh et al. (2014) reported higher enzyme yield by *Aspergillus niger* using sugarcane bagasse as compared to rice bran, wheat bran and sesame oil cake.

Our results are not in agreement with those of Sreedevi and Reddy (2012) who used different agricultural residues like wheat

bran, rice bran, Bengal gram bran, red gram bran, groundnut oil cake, sesame oil cake, coconut oil cake, cotton oil cake, soyabean meal, oatmeal, corn meal and barley meal in order to study their effect on phytase production under submerged fermentation and reported wheat bran as best substrate.

The results obtained with rice bran as carbon source are best as compared to other carbon sources used. It might be due to the reason that rice bran provided adequate amounts of nutrients like carbohydrates, proteins, fats, calcium, phosphorous, potassium and amino acids in the presence of oxygen supply. These nutrients are necessary for the adequate production of phytase. The results of our study clearly established the fact that phytase production is growth associated as well and fermentation performed above or below the optimum growth temperature, pH and substrate concentration significantly affects the enzyme yield.

It was found that selected fungal isolate produced maximum enzyme and biomass at optimized physical and chemical parameters. Our results are in agreement with Lata et al, (2013) who optimized culture conditions for the production of phytase from *Aspergillus heteromorphus*. In other studies, authors reported that phytase yield and biomass formation were strongly correlated indicating strong growth associated phytase production by selected fungal strains *Mucor racemosus*, *Rhizopus oligosporus*, *Aspergillus niger* (Roopesh *et al*., 2005; Sabu *et al*., 2002; Krishna *et al*., 2001)

Maximum phytase production was noted at optimum temperature, pH and concentration of each substrate. Order of substrate choice for highest phytase production is rice bran > wheat bran > oat bran. It is concluded that rice bran supported maximum fungal growth and higher enzyme production as compared to wheat bran and oat bran. Thus rice bran proved best substrate to produce phytase on larger scale. The results of our study indicated that maximum enzyme is produced at optimized temperature & pH and substrate concentration significantly affects the enzyme yield.

Conclusion:

Optimization of culture conditions enhanced phytase production and indigenous strain of *A. flavus* PHY168 can be used for cost effective phytase production to meet its demands in food and feed industries.

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