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Optimization of phytase production in solid state fermentation by different fungi

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The present study was conducted to explore the potential fungal isolates for maximum production of phytase by optimizing solid substrate preference and growth parameters (pH, temperature, fermentation time) of solid state fermentation (SSF). It has been inferred from the results that all the tested fungi (*Aspergillus niger, Aspergillus flavus, Aspergillus versicolor, Aspergillus nidulans, Cladosporium cladosporioides, Trichoderma reesei,* and *Trichoderma viride*) showed inducible expression of phytase. Fungal isolates showed different preference of solid substrate out of wheat bran, lentil, oat, corn and bagasse for maximum production of phytase. Lentil and bagasse were not used preferably by any tested fungi. Among the tested fungi, maximum phytase production was observed in *A. flavus* (80 U/g of solid substrate) using wheat bran as solid substrate at pH 6 after 7 days of fermentation period at 30°C. It was established that solid substrates with high phytate and low inorganic phosphate (P*i*) contents are substrate of choice for phytase production by SSF.

Key words: Phytase, solid state fermentation, optimization.

INTRODUCTION

Cereals, legumes and oilseed crops are cultivated globally over approximately 90% of agricultural land and serve as major source of nutrients for animal kingdom (Reddy et al., 1982). In these plants, more than 80% of the total phosphorous is stored in the form of phytate (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, IP_6) molecules (Spier et al., 2008). Phytate (phytic acid) has highly stable structure and differs from other organo-phosphates because of its high phosphate content (Kerovuo et al., 2000).

Monogastric animals poorly digest phytate and excrete most of it in fecal waste. While passing through the digestive track, the indigestible phytate exerts strong anti-nutritive effects such as chelation of essential minerals like iron, calcium, zinc and magnesium resulting in insoluble phytate mineral complexes. These complexes limit the bioavailability of essential minerals (Lopez et al., 2002). It is also reported that binding of phytic acid with dietary proteins and amino acids reduces their digestibility and the functionality of digestive enzymes like trypsin, and amylase (El-Batal and Karim, 2001; Li et al., 2008).

In animal feeding industries, feed is usually supplemented with inorganic phosphate in order to meet the phosphorous need for proper growth and development of animals. However, anti-nutritional effects of phytate remained unaffected (Sandberg and Andlid, 2002). Excretion of indigestible phytate having large amount of phosphorous in manure leads to redistribution of phosphorous in soil (Turner and Leytem, 2004). It may leach in waterways and execute eutrophication that generates water quality issues. Hence, elevated level of phosphorous in water and soil also creates several environmental problems.

To avoid the phytate related issues, there is need to introduce the methods for degradation of phytate. The

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physical and chemical methods are expensive and reduce the nutritional value of feed as well (Khetarpaul and Chauhan, 1990). Therefore, enzymatic degradation of phytate appears to be of great interest. Phytase (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.38 and EC 3.1.3.26) hydrolyzes phytate stepwise into lower inositol phosphates, myo-inositol and inorganic phosphate (Kim and Lei, 2005).

The use of phytase in animal industry is not only to enhance the utilization of phosphorous, but it also eliminates the anti-nutritive effects of phytate which ensure the bioavailability of minerals and phosphorous to promote bone strength (Lei et al., 2011). It also reduces the fecal phosphorous contents up to 50% (Konietzny and Greiner, 2004). Therefore, phytases are considered as potential candidate for removing some of the negative environmental impacts exerted due to livestock production (Hegeman and Graban, 2001). The phytase is also gaining attention of animal industries for the degradation of phytate present in animal feeds (Li et al., 2008).

Phytases are present naturally in plants, animals and microbes. The activity of animal phytases is minimal in comparison to the plant and microbial phytases (Weremko et al., 1997). The intrinsic phytases of some plants, such as wheat and barley, have high activity but their pH spectrum (4-7.5) is narrow. These enzymes are also more heat labile (Greiner and Konielzny, 2006).

The present study is designed to optimize the nutritional and physical parameters of solid state fermentation for the maximal production of extracellular phytase from various fungi.

MATERIALS AND METHODS

Preparation of inoculum

All the fungi (*A. niger, A. flavus, A. versicolor, A. nidulans, Cladosporium cladosporioides, T. reesei,* and *T. viride*) used in the present study were obtained from the Fungal Bank of Institute of Agricultural sciences, University of the Punjab, Lahore, Pakistan. The cultures were grown and maintained on malt extract agar slants composed of 2.5% malt extract, 0.5% yeast extract, 0.5% glucose and 2% agar. After sterilization, the slants were inoculated with respective fungus and incubated at 30°C for growth for seven days. After growth, the slants were stored at 4°C until further use.

Seven days old fully sporulated slants were used for the preparation of inoculum by suspending the fungal spores in 10 ml of 0.1% Tween-80 separately. The spore suspension was adjusted to $1x\ 10^7$ spores per ml and 100 µl of spore suspension was used to inoculate the media for respective fungi unless otherwise mentioned.

Qualitative screening of fungal strains for phytase production

The fungal strains were qualitatively screened for extracellular phytase production using phytase screening medium (PSM) containing glucose (2%), tryptone (1%), NaCl (0.5%), KCl (0.1%), potassium phytate (0.2%) and agar (2%). The PSM plates were inoculated and incubated at 30°C for 3 days. The plates were observed for zone of clearance around fungal growth. The zone forming fungi were selected for quantitative screening.

Quantitative screening

The qualitatively positive fungal cultures were checked for the expression of phytase enzyme whether it is constitutive or inducible. Each fungal strain was grown on phytase screening broth and in the medium including K_2HPO_4 (0.5%) instead of potassium phytate. The media was inoculated with fungal strains in 250 ml Erlenmeyer flasks and incubated at 30°C for 5 days. The mycelial mat was removed by passing the suspension through two layers of muslin cloth. The filtrate was centrifuged at 9447 g for 20 minutes at 4°C. The cell free supernatant was used for phytase assay. In this assay, the expression of phytase enzyme was observed inducible in all the tested fungi.

Substrates

The substrates used for phytase production by solid substrate fermentation (SSF) includes: wheat bran, lentil, oat, corn and bagasse. The substrates were purchased from the local market of metropolitan city of Lahore, Pakistan. The phytic acid contents of these substrates were used as inducer for phytase expression.

Optimization of phytase production by solid substrate fermentation (SSF)

The nutritional and physical parameters of SSF were optimized by changing one parameter at a time to get maximal production of phytase from each fungus. The physical parameters included pH (4-9), temperature (20-40°C) and incubation time (24-192 h).

The medium for SSF was prepared by adding 2.5 g of finely ground substrate (180 µm particle size) in 250 ml Erlenmeyer flask supplemented with 5 ml of nutrient solution (0.2% glucose, 1% tryptone, 0.5% NaCl, 0.1% KCl) and sterilized at 121°C under 15 psi for 20 min. After cooling, fermentation medium was inoculated and initially incubated at 30°C for 5 days. Phytase production was evaluated by phytase assay.

Preparation of enzyme extract

In order to prepare the enzyme extract, 10 ml of 0.2 M potassium acetate buffer was added in each fermented flask and incubated at 4°C for 24 h in orbital shaker at 200 rpm. The mycelial mat was removed by passing the suspension through two layers of muslin cloth. The filtrate was centrifuged at 9447 *g* for 20 min at 4°C. The clear supernatant was used as crude enzyme extract for phytase assay.

Phytase assay

Phytase activity was determined spectrophotometrically by measuring the inorganic phosphorous released from the substrate as described by Harland and Harland (1980).

One unit of phytase activity is defined as ''the amount of phytase required to liberate 1 µmole of inorganic phosphorous per unit time under assay conditions.''

RESULTS AND DISCUSSION

The phytase is gaining importance at industrial level and has become an object of extensive research because it is involved in the breakdown of phytate which is associated with several problems of the animal industry and environ-

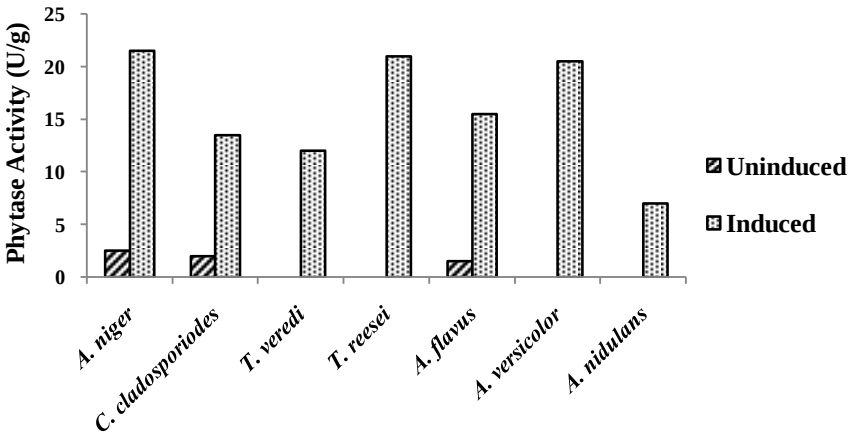

Figure 1. Phytase production under uninduced and induced conditions using KH_2PO_4 as inducer.

mental pollution. In order to ensure the maximal enzyme yield at low cost, the selection of microorganism, culture conditions, substrate selection and physical conditions are critical which play key role in enhancing the enzyme production (Vats and Banerjee, 2004; Sasirekha et al., 2012).

In the present study, a correlation between solid substrate, physical parameters of SSF and production of phytase from fungi has been studied.

Inducible expression of phytase

The fungi showing zone of clearance around its growth circle were selected for qualitative screening. In qualitative screening, the selected fungi were assessed for phytase production under induced and uninduced conditions. In uninduced condition, KH_2PO_4, while in case of induction conditions potassium phytate, were used as sole sources of phosphorous. The results show that all tested fungi had inducible expression of phytase (Figure 1). The results are similar to the work of Sasirekha et al. (2012) who have reported expression of phytase only in media including phytate as source of phosphorous and negligible expression, when inorganic phosphate is added in media.

Optimization of SSF parameters

For the production of phytase and other metabolites from filamentous fungi, SSF is preferred over submerged fermentation because substrate provides physical support and energy for fungal growth (Pandey et al., 2001). In the present study, five solid substrates including wheat bran, corn, oat lentil and bagasse were evaluated for phytase production. These substrates served both as source of energy and as inducer for phytase production due to their phytic acid contents. During optimization of substrates, the pH was adjusted to 7 and fermentation was performed at 30°C for 5 days. The substrate preference of tested fungi for maximum phytase production is given in Figure 2. The results indicate that lentil and bagasse are not preferably used in SSF by all fungi to produce phytase. It was interesting to note that tested fungi showed different preferences for solid substrate for maximal production of phytase. These results do not corroborate with many other reports which supports only wheat bran as potential substrate for phytase production (Salmon et al., 2012; Sreedevi and Reddy, 2012; Pandey et al., 1999) whereas, the studies of Roopesh et al. (2006); Bogar et al. (2003), Spier et al. (2008) and Selvamohan et al. (2012) support our finding that other solid substrates may also be considered as good candidate for phytase production. However, this observation is dependent on the type of microorganism used for SSF. It has been reported that phytate contents (% of dry weight) of lentil (0.27%) and bagasse (0.038%) are low in comparison to the wheat bran (3.29%), oat (1.37%) and corn (1.05%) (Coulibaly et al., 2011; Mittal et al., 2012). In the present study, it was observed that substrates with high phytate and low inorganic phosphate (P*i*) content are preferred over the substrates with low phytate and high inorganic phosphate (P*i*) contents for production of phytase. Hence, it is suggested that the substrates with high phytate and low P*i* are more preferable for phytase production by SSF, which is also supported by the studies of Vats and Banerjee (2004).

Physical parameters significantly affect the growth of microorganism as well as the growth associated production of desirable metabolite in SSF. In the present study, physical parameters (pH, temperature and incubation time) were optimized using standardized solid substrate specific for each fungus to get the maximum phytase production.

In order to optimize pH for phytase production from

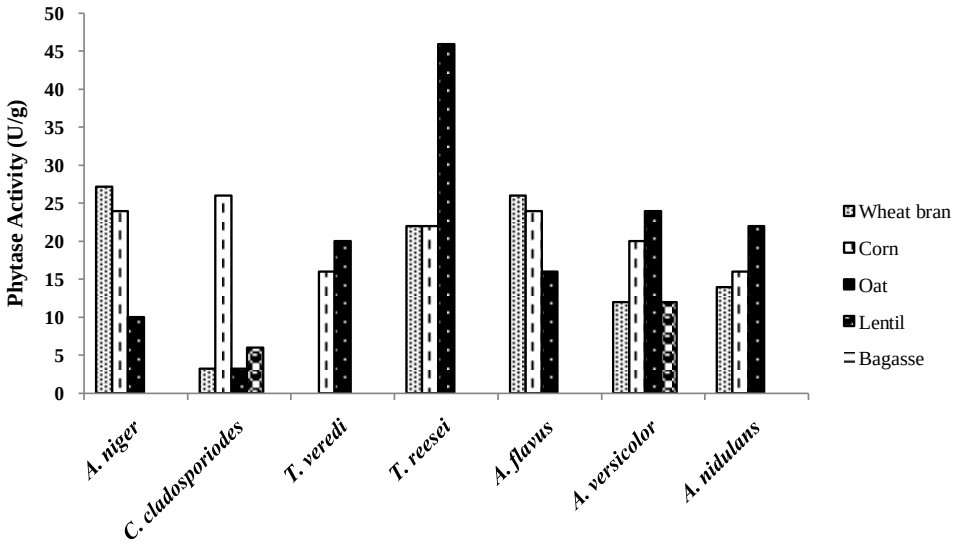

Figure 2. Optimization of solid substrates for phytase production.

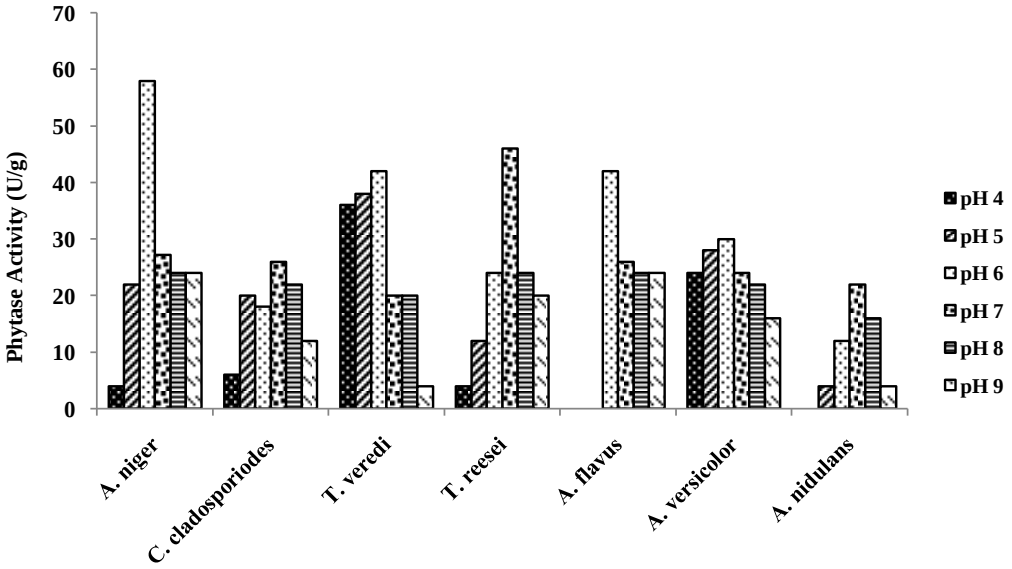

Figure 3. Optimization of pH for phytase production from tested fungi using SSF on specific substrates: Wheat bran for *Aspergillus niger* and *Aspergillus flavus*; Oat for *Trichoderma reesei*, *Trichoderma viride*, *Aspergillus versicolor* and *Aspergillus nidulans*; Corn for *Cladosporium cladosporioides*.

tested fungi, the pH of media was varied from 4 to 9 and SSF was conducted for 5 days at 30°C. The results show that the optimum pH of all tested fungi falls in pH ranging from 6 to 7. *A. niger* (58 U/g), *A. flavus* (42 U/g), *T. viride* (42 U/g) and *A. versicolor* (30 U/g) had the optimum pH 6 while *T. reesei* (46 U/g), *C. cladosporioides* (26 U/g) and *A. nidulans* (22 U/g) have pH 7 (Figure 3). These findings corroborate several investigations that report that acidic to neutral pH of fermentation medium favors 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; Selvamonan et al., 2012).

Using optimized substrate type and pH, SSF was performed at different temperatures for 5 days to standardize optimum temperature of all tested fungi for phytase production. It is observed that all tested fungi show maximum phytase production at their optimal growth

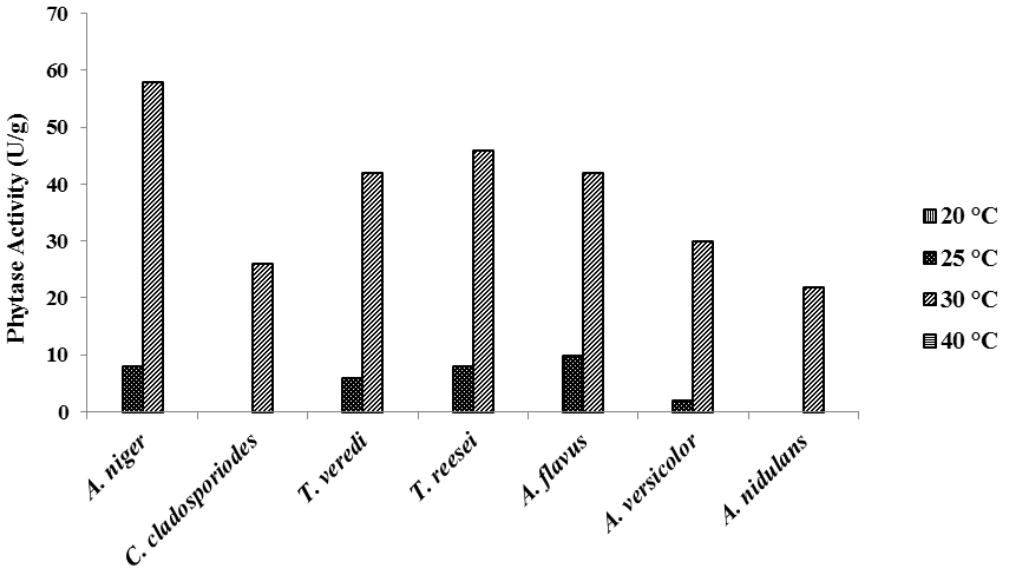

Figure 4. Optimization of temperature for phytase production by SSF.

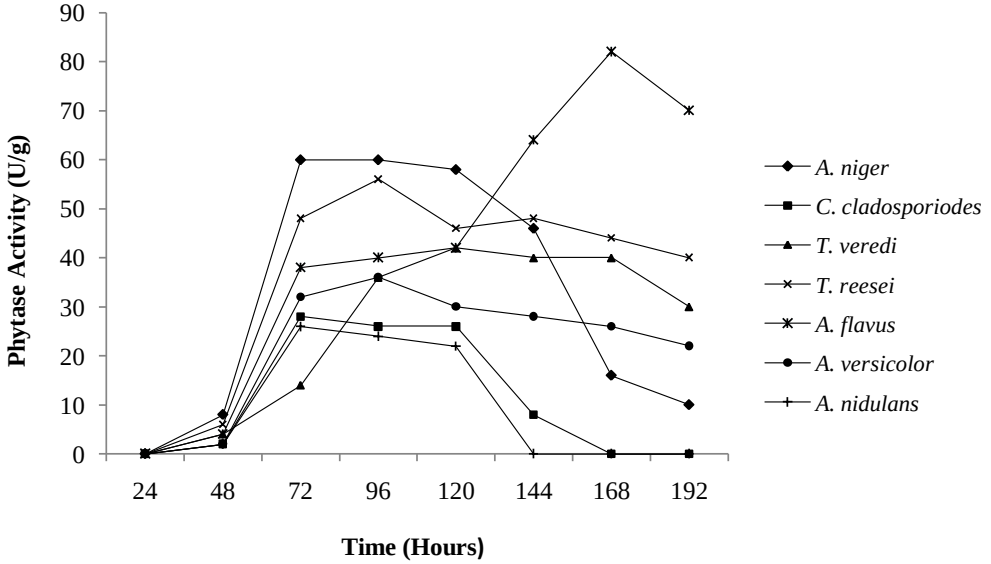

Figure 5. Optimization of fermentation time for phytase production from tested fungi using SSF.

temperature (30°C) (Figure 4), which corroborates other studies (Al-Asheh and Duvajak, 1995). Fungi produce phytase maximally at their optimal growth 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.

In order to standardize the time interval for optimal phytase production, SSF was performed for different time intervals using standardized substrate, pH and temperature for each fungus. It was observed that each tested fungus showed optimal phytase production after specific time intervals of fermentation. The maximum phytase production was observed in *A. flavus* (80 U/g) after 7 days of SSF (Figure 5).

Finally, it was concluded that *A. flavus* is a potential candidate for phytase production using SSF. The culture conditions significantly affect the enzyme production. SSF performed using standardized nutritional and physical parameters, which are specific for each fungus improves the enzyme yield. The fungal cultures showed differential preference for solid substrates and the solid substrate with high phytate: P*i* ratio is more preferable for phytase production.

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