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SOLID STATE CULTURING OF THERMOPHILIC FUNGI FOR PHYTASE PRODUCTION

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Abstract

Seven different types of themophilic fungi such as *Aspergillus fumigatus*, *Humicola insolens, Rhizomucor miehei*-I & II, *Sporotrichum thermophile, Thermomyces lanuginosus*-I & II were isolated from the compost of various localities. Among all isolates, *Sporotrichum thermophile* was found to be the best isolate for the phytase production. Six different types of solid materials (wheat bran, rice bran, rice husk, fish meal, corn seed and corn gluten) were evaluated as growth substrate for phytase production by *Sporotrichum thermophile.* Of all the sources tested, wheat bran supplemented with diluent containing (g/L); $(NH_4)_2SO_4$; 5.0, KH_2PO_4; 1.0, Yeast extract; 2.0 gave maximum production (4.16 U/mL/min) when 4% volume of the 250 mL conical flask was used after 96 hrs conidial inoculation at 45^oC using solid-state fermentation.

Introduction

In modern age of biotechnology, enzymes have proved their market demand over other products of biotechnology with annual sales close to 2.0 billion dollars. Phytases (EC 3.1.3.8), phosphatases that catalyze the hydrolysis of phosphate moieties, have a big share in enzyme business due to its widespread application as a feed supplement (McCoy, 1998; Vohra & Satyanarayana, 2003; Greiner & Konietzny, 2006). The phytases enhance the bioavailibility of minerals, protein and phosphorus in monogastric animals. They reduce the phosphorus pollution in areas of intensive livestock production (Wodzinski & Ullah, 1996). The thermostability of phytase suggests potential biotechnological applications in the pulp and paper industry as a biological agent to remove plant phytic acid. The enzymatic degradation of phytic acid will not produce toxic by-products, so it is environmental friendly (Ciofalo *et al*., 2003).

A large number of microbes including bacteria, yeast and filamentous fungi have been used for phytase production. Selection of particular microbe depends on the nature of substrate, environmental conditions and desired final product. Thermophilic fungi have complex or unusual nutritional requirements and well-known microbes to produce phytase (Pandey *et al*., 2001; Bogar *et al*., 2003; Chadha *et al*., 2004; Singh & Satyanarayana, 2006).

Solid-state fermentation (SSF) system has generated much interest in recent years because it offers several economical and practical advantages including high product concentration, improved product recovery, simple cultivation equipment and lower plant operational cost (Becerra & Siso, 1996; Pandey *et al*., 2001). In view of increasing demand for phytase it is essential to produce phytase in a cost-effective manner. Phytase required for commercial feed preparation must meet following specifications i.e., thermostability and activity over wider pH range, which is only possible with thermophilic fungi. Therefore, the present study was conducted with the aim to isolate a potent strain of thermophilic fungi from local habitat and optimize after screening for the production of phytase.

Materials and Methods

Isolation of organisms: Different cultures of thermophilic fungi were isolated from different compost and soil samples of Lahore by serial dilution method after Clark *et al*., (1958). The soil samples were collected in sterile polythene bags. One gram of sample was suspended in 100 mL of sterilized distilled water. The soil suspension was further diluted up to 10^4-10^6 times. One millilitre of this dilute suspension was then transferred to individual Petri plates containing potato dextrose agar medium. The fungal cultures were further purified from bacterial contaminants by using 10 mg/L combination of penicillin and streptomycin (1:1 ratio) in the Petri plate medium. All the isolates of thermophilic fungi were identified by microscopic examination after Cooney & Emerson (1964); Domsch *et al*., (1980); Onion *et al*., (1986) and Koneman *et al*., (1991). Independent colonies of each identified isolate were picked up and transferred to potato dextrose agar (PDA) slants for culture maintenance. The cultures were stored in a refrigerator at 4°C for further studies.

Inoculum: The conidia from 3-5 days old slant culture were wetted by adding 10 mL of sterilized 0.005% Monoxal O.T (diacetyl ester of Sodium sulphosuccinic acid) to each slant. The conidia were scratched with sterilized inoculating needle and the tubes were shaken gently to break the clumps of conidia. Conidial suspension was used as an inoculum. Inoculum size was measured by measuring the density of conidia (number of conidia per unit volume) with Haemacytometer, Neubauer improved; precicdor HBG, Germany (Tiefe depth profondeur 0.10 mm and 0.0025mm^2 area).

Fermentation: Solid-state fermentation was carried out in the present studies. Ten grams of solid substrate was transferred to a 250 mL conical flask and moistened by adding 10 mL of diluent. The flasks were cotton plugged and sterilized in an autoclave. The flasks were cooled to room temperature and were then inoculated with 1.0 mL conidial suspension. The substrate cultures were incubated at 45 $\pm$ 2ºC for 72 hrs. The flasks were shaken twice a day. All the experiments were run parallel in triplicate. After specific time of fermentation, 50 mL of 2.0% aqueous solution of $CaCl_2.2H_2O$ was added to each flask. Flasks were put in a rotary shaker operated at 200 rpm for 2 hrs at room temperature for the extraction of enzyme from fermented mass (Ebune *et al.,* 1995). The suspension was squeezed and it was centrifuged at 6000 x g for 20 minutes at 4^{o}C. The clear supernatant was used for enzyme titer.

Phytase assay: Phytase activity was assayed after some modification of Harland & Harland (1980) and Heinonen & Lahti (1981) methods using Sodium phytate as substrate and the inorganic phosphorus released was measured spectrophotometerically by using the Taussky-Schoor reagent. Half milliliter of Sodium phytate (0.00682M) was added to 0.1 mL of $MgSO_4$ (0.05M) and 0.1mL of Sodium acetate buffer (0.2M). Enzyme solution (0.1 mL) was added to above mixture and the mixture was incubated at 50^{o}C for 30 minutes. After incubation, 1.0 mL of 10% tricarboxylic acid (TCA) was added along with 2.0 mL of distilled water. Mixed well and 5.0 mL of Taussky-Schoor reagent was added. Taussky-Schoor reagent was prepared when 10.0 g Ammonium molybdate was mixed with 10.0 mL H_2SO_4 (10N) and further diluted with 70.0 mL of deionized water. Then 5.0 g of ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) was added and made the final volume upto 100.0 mL. Absorbance was measured at 660 nm by using spectrophotometer and

liberated inorganic phosphate was estimated after comparing the absorbance with known concentration of KH_2PO_4 using same assay conditions instead of enzyme. One unit of phytase activity is defined as "the amount of enzyme that liberates one μmol of inorganic phosphate at temperature (50^oC) and pH (5)".

Statistical analysis: Duncan's multiple range tests in the form of probability <*p*> values were used to find out the significant difference among replicates. Treatment effects were compared after Snedecor & Cochrane (1980) using computer software Costat, 3.03 Berkeley, CA 94701.

Results and Discussion

Seven strains of five different thermophilic fungi such as *Aspergillus fumigatus*, *Humicola insolens, Rhizomucor miehei*-I & II, *Sporotrichum thermophile, Thermomyces lanuginosus*-I & II were isolated from compost soil and were screened for phytase production. *Aspergillus fumigatus* produced 0.04 U/mL/min of phytase while *Humicola insolens, Rhizomucor miehei*-I & II, *Sporotrichum thermophile, Thermomyces lanuginosus*-I & II gave 0.22, 0.20, 0.76, 2.20, 0.20 and 0.53 U/mL/min respectively. Of the seven thermophilic fungal strains screened for phytase production, *Sporotrichum thermophile* was found to produce higher extracellular phytase when grown on solid state wheat bran (Table 1). Chadha *et al*., (2004) isolated and screened out thermophilic fungi and found nine thermophilic strains having the potential of phytase production like *Rhizomucor pusillus, Humicola grisea, Sporotrichum thermophile*, *Humicola insolens, Thermomyces lanuginosus*-I, *Thermomyces lanuginosus*-II, *Rhizomucor miehei*-I, *Rhizomucor miehei*-II and *Aspergillus fumigatus.* Singh & Satyanarayana (2008) also investigated that *Sporotrichum thermophile* has the potential for enhanced production of phytase.

The main product of a fermentation process often determines the choice of carbon source, particularly if the product results from the direct dissimilation of it. It is common practice to use carbohydrates as carbon source in microbial fermentation processes. The most widely available carbohydrates for solid-state fermentation are the agricultural by-products, such as wheat bran, rice husk and rice bran. These substrates serve not only as the source of carbon but also provide organic nitrogen (amino acid), vitamins and minerals. The majority of enzymes which are of industrial interest are inducible, the inducible character of such enzymes need the screening of such substrates (Whitaker, 1973). Different carbon sources like wheat bran, rice husk, rice bran, fish meal, corn seed and corn gluten were explored for the production of phytase. The enzyme activity for wheat bran (2.21 U/mL/min), rice husk (2.15 U/mL/min), rice bran (0.32 U/mL/min), fish meal (0.25 U/mL/min), corn seed (0.03 U/mL/min) and corn gluten (0.01 U/mL/min) was calculated. The results obtained with wheat bran as carbon source are best as compared to other carbon sources used, which gave 2.21 U/mL/min of phytase and was selected for subsequent studies (Table 2). It might be due to the reason that wheat 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. These findings are of particular importance because the use of agricultural residues are more feasible than that of purified substrates (Lynd *et al*., 2002).

Table 1. Potential of different thermophilic fungi for phytase production.

Organism	Phytase activity (U/mL/min)
Sporotrichum thermophile	2.20 ± 0.1^{a}
Thermomyces lanuginosus-I	0.20 ± 0.04^{d}
Thermomyces lanuginosus -II	0.53 ± 0.02^{c}
Rhizomucor miehei-I	0.20 ± 0.01^{d}
Rhizomucor miehei-II	0.76 ± 0.06^{b}
Humicola insolens	0.22 ± 0.02^{d}
Aspergillus fumigatus	0.04 ± 0.01^{e}
LSD	0.062

Each value is mean average of three parallel replicates. ± indicate standard deviation among replicates. Numbers followed by different letters differ significantly at $p \leq 0.05$. Fermentation time = 72 hrs, Carbon source = Wheat Bran, Diluent = M-I

Table 2. Effect of different carbon sources on phytase production by *Sporotrichum thermophile*.

Carbon source	Phytase activity (U/mL/min)
Corn seed	0.03 ± 0.03^{e}
Rice husk	2.15 ± 0.002^{b}
Corn gluten	0.01 ± 0.07^{f}
Fish meal	0.25 ± 0.002^{d}
Wheat bran	2.21 ± 0.007^{a}
Rice bran	0.32 ± 0.007^{c}
LSD	0.01

Each value is mean average of three parallel replicates. ± indicate standard deviation among replicates. Numbers followed by different letters differ significantly at $p \leq 0.05$. Fermentation time = 72 hrs, Diluent = M-I

All micro-organisms require certain mineral elements for growth and metabolism (Hughes & Poole, 1989, 1991). There is obviously a need for batch analysis of medium components to ensure that the assumption can be justified. In the present studies three different media consisting of various compositions and concentrations of minerals/ compounds/extracts used as diluents such as M-I, M-II, M-III were compared with tap and distilled water separately such as M-IV and M-V for the production of phytase. The enzyme activity estimated with these was 2.22 U/mL/min., 1.46 U/mL/min., 1.46 U/mL/min., 0.18 U/mL/min., and 0.06 U/mL/min., respectively. Diluent (M-I) containing 0.1g/100mL KH_2PO_4, 0.2g/100mL yeast extract, 0.5g/100mL $(NH_4)_2SO_4$ was optimized as the best combination of nutrients for *Sporotrichum thermophile* to produce higher level of phytase i.e., 2.22 U/mL/min., (Fig. 1). It might be due to the reason that M-I salts combination has a balanced proportion of macro and micro nutrients, required by the organism for growth and subsequent enzyme production, along with wheat bran. Other diluents may be lacking in any of the components essential for the fungal growth and enzyme synthesis. M-I diluent contains suitable amount of KH_2PO_4 (1.0 g/L) which gave optimum phytase level. Thus, the production enhanced with the incorporation of inorganic phosphate as described by Vohra & Satyanarayana (2003) & Vats & Banerjee (2004).

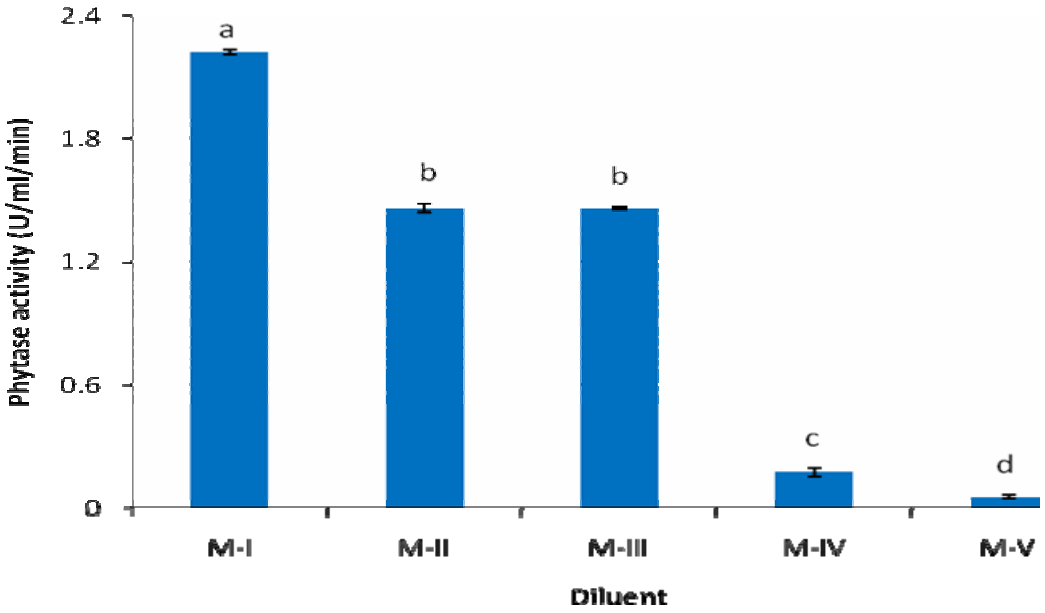

Fig. 1. Effect of diluents on phytase production by *Sporotrichum thermophile.* Each value is mean average of three parallel replicates. ± indicate standard deviation among replicates. Letters differ significantly at p≤0.05. Fermentation time = 72 hrs, Carbon source = Wheat Bran

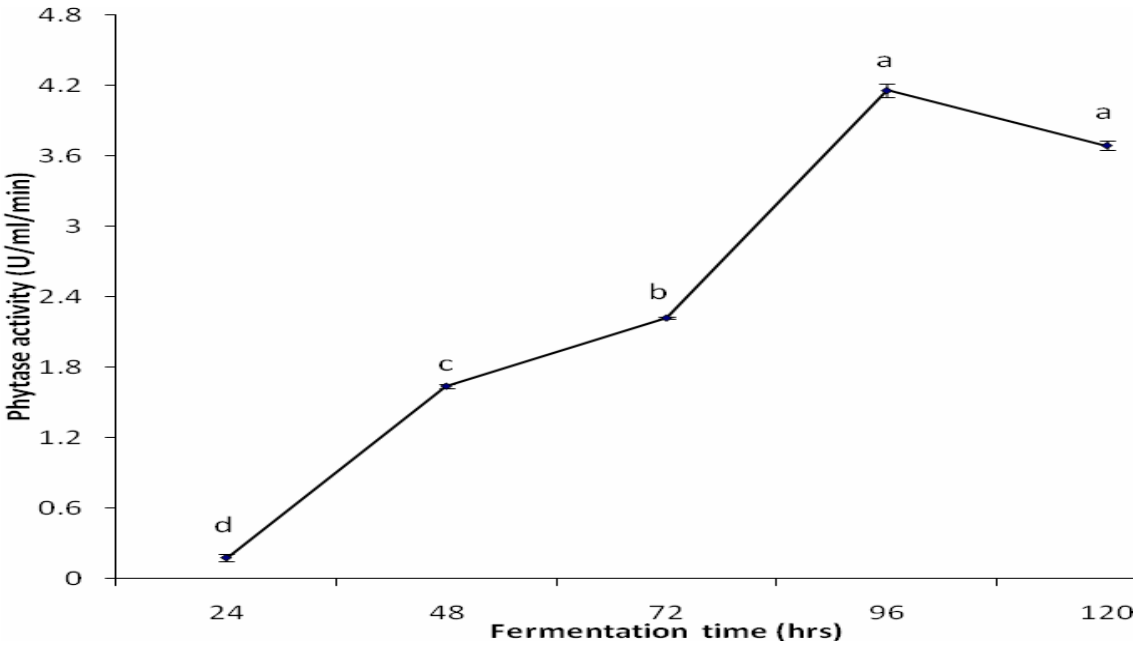

Fig. 2. Effect of fermentation time on phytase production by *Sporotrichum thermophile.* Each value is mean average of three parallel replicates. ± indicate standard deviation among replicates. Letters differ significantly at p≤0.05. Carbon source = Wheat Bran, Diluent = M-I

The production of phytase by *Sporotrichum thermophile* with different incubation time i.e., 24, 48, 72, 96 and 120 hrs after inoculation was carried out using solid state fermentation. Result showed that maximum phytase titer (4.158 U/mL/min) was achieved after 96 hrs, further increase in incubation time has a negative effect on phytase production (Fig. 2). This might be due to the reason that substances were more prone towards the synthesis of enzyme in the beginning while with the passage of time they became less susceptible. And with the prolonged incubation times, the portions became hydrolyzed by microbes which inhibited the enzyme secretion path way (Saha, 2003).

Table 3. Effect of depth of medium on phytase production by *Sporotrichum thermophile*.

Weight of solid residue in 250mL flask (g)	Phytase activity (U/mL/min)
10	4.15 ± 0.01^{a}
15	3.07 ± 0.04^{b}
20	2.08 ± 0.09^{c}
25	1.065 ± 0.04^{d}
30	0.76 ± 0.04^{e}
35	0.62 ± 0.04^{f}
40	0.42 ± 0.03^{g}
LSD	0.08

Each value is mean average of three parallel replicates. ± indicate standard deviation among replicates. Numbers followed by different letters differ significantly at $p \leq 0.05$. Fermentation time = 72 hrs, Carbon source = Wheat Bran, Diluent = M-I

The consideration of the stoichiometry of respiration gives an appreciation of the problem of oxygen supply; it gives no indication of an organism's true oxygen demand as it does not take into account the carbon that converted into biomass and products. However, it is inadequate to base the provision of oxygen for a solid state fermentation in conical flask. Production of phytase by *Sporotrichum thermophile* at different wheat bran concentrations i.e., 10g, 15g, 20g, 25g, 30g, 35g, 40g was carried in 250 mL conical flask. The enzyme activity with these concentrations was estimated as 4.15 U/mL/min, 3.07 U/mL/min, 2.08 U/mL/min, 1.06 U/mL/min, 0.76 U/mL/min, 0.62 U/mL/min and 0.42 U/mL/min respectively (Table 3). In solid-state fermentation, it is optimized that one fourth of the available space of the vessel (conical flask) is suitable for better yield of enzymes.

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