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Thermostable Phytase Production by *Thermoascus aurantiacus* in Submerged Fermentation

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Abstract

Phytases act on phytic acid, an antinutrient factor present in animal feeds, and release inorganic phosphate. We optimized the production parameters for phytase production using *Thermoascus aurantiacus* (TUB F 43), a thermophilic fungal culture, by submerged fermentation. A semisynthetic medium containing glucose, starch, peptone, and minerals supplemented with 3.75% (w/v) wheat bran particles was found to be the best production medium among the various combinations tried. Further supplementation of this medium with surfactants such as Tween-20 and Tween-80 considerably enhanced the enzyme yield. A maximum phytase activity (468.22 U/mL) was obtained using this production medium containing 2% (v/v) Tween-20 after 72 h of fermentation at 45°C in shake-flask cultures with a rotation of 150 rpm. Herein we present details of a few of the process parameter optimizations. The phytase enzyme was found to be thermostable, and the optimal temperature for phytase activity was found to be 55°C. However, 80% of the activity still remained when the temperature was shifted to 70°C.

Index Entries: *Thermoascus aurantiacus*; thermostable phytase; submerged fermentation; surfactants; phytic acid.

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Introduction

Phytic acid is the major storage form of phosphate in cereal grains, legumes, and oilseeds, which are the principal components of animal feeds. Phytate constitutes 1–4% by weight of cereals and oilseeds, and it is the primary phosphorus and myoinositol reserve in most seeds and usually accounts for 60–90% of the total phosphorus *(1)*. Monogastric animals such as chickens and pigs are unable to metabolize phytic acid and thus create serious environmental pollution owing to the excretion of high-phosphate-containing manure. The phosphate moieties of phytic acid chelate multivalent cations such as Zn^{2+}, Mg^{2+}, Ca^{2+}, and Fe^{2+} and decrease their bioavailability *(2,3)*. The insoluble phytate also binds to proteins, thus rendering the vital nutrients such as amino acids unavailable *(4)*. Therefore, the presence of phytic acid in animal feeds for chickens and pigs is undesirable, and livestock feed must be supplemented with inorganic phosphate.

Phytase or myoinositol hexakisphosphate hydrolase (EC 3.1.3.8) belongs to the group of phosphoric monoester hydrolases, and catalyzes the hydrolysis of myoinositol hexakisphosphate to inorganic monophosphate and lower phosphoric esters of myoinositol or, in some cases, to free inositol *(5)*. Phytases are present in plants, certain animal tissues, and microorganisms *(6)*. Phytase activity in microorganisms has been found most frequently in fungi, in particular *Aspergillus* sp. such as *Aspergillus ficcum*, and *Aspergillus niger (7,8)*. It occurs also in bacteria such as *Bacillus* sp. and *Escherichia coli (9,10)* and in yeast such as *Saccharomyces cerevisiae (11)*. Supplementation of animal feedstuff with phytases can enhance the bioavailability of phosphate and thus reduce the unutilized dietary phosphorous pollution in concerned areas of animal agriculture. However, a major barrier to the wide use of phytase is the constraint of thermal stability required for this enzyme to withstand heat inactivation during feed processing. The currently available industrial phytases mainly originate from *A. niger* and have low intrinsic resistance to heat inactivation *(12)*. Hence, there is a definite need for second-generation phytases with improved properties such as higher thermostability and catalytic efficiency. Enzymes from thermophilic organisms are generally more stable and better withstand proteolysis and are more resistant to mechanical denaturation than those produced by mesophiles. Submerged as well as solid-state fermentation was employed for the production of phytase *(13–15)*. Our objective was to optimize the various process parameters for the production of an active thermostable phytase by submerged fermentation (SmF).

Materials and Methods

Fungal Strain

Thermoascus aurantiacus (TUB F 43), a thermophilic fungal strain obtained in freeze-dried form from Technical University, Budapest, was activated and used. TUB F 43 has the following accession no. at the American Type Culture Collection (ATCC), Manassas, VA: ATCC 58156.

Maintenance of Culture

Potato dextrose agar (PDA) medium was used for maintaining the cultures. The PDA slants were streaked with the fungal culture and incubated at 45°C for 7 d for sporulation. The fully sporulated slants were stored at 4°C for further use.

Preparation of Inoculum

To a fully sporulated PDA slant, 10 mL of sterile distilled water containing 0.1% Tween-80 was added, and spores were dislodged using an inoculation loop under aseptic conditions. Ten milliliters of this spore suspension was used as the master spore suspension, and it was appropriately diluted for required density of spores. The pour plate followed by colony count determined the number of viable spores in the inoculum.

Submerged Fermentation

The basic medium used was a semisynthetic type having the following composition: 2.8 g/L of starch, 0.5 g/L of glucose, 1.8 g/L of peptone, 0.05 g/L of KCl, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of KH_2PO_4, and 0.2 g/L of $CaCl_2 \cdot H_2O$ (pH 5.5). The medium was further modified by adding either wheat bran extract (% [v/v]) or wheat bran particles (% [w/v]) for further studies.

Forty milliliters of the desired medium was placed in 250-mL Erlenmeyer flasks and sterilized by autoclaving at 121°C for 20 min. After cooling, the medium was inoculated with 1 mL (6×10^7 spores) of inoculum. The flasks were incubated at 45°C on a rotary shaker at 150 rpm. The samples were withdrawn as whole flasks at desired time intervals.

Preparation of Wheat Bran Extract

Fifty grams of wheat bran was transferred into a container, 400 mL of distilled water was added, and the mixture was cooked for 30 min. The boiled mixture was filtered through muslin cloth and gently squeezed by hand in order to obtain the complete extract of wheat bran.

Enzyme Extraction and Estimation of Biomass

After incubating the whole culture for a desired period of time, 40 mL was centrifuged at 3700*g* for 15 min. The supernatant was separated out and used as the crude enzyme. The total wet biomass was dried in a hot-air oven at 80°C for 12 h, and the amount of biomass was expressed in milligrams (dry weight)/milliliter of culture.

Enzyme Assay

Phytase activity was assayed by measuring the inorganic phosphorus released from sodium phytate as per the method described by Harland and Harland *(16)*. One unit of enzyme activity was defined as the amount

of phytase required to release 1 µg of phosphorus/min under assay conditions.

Optimization of Process Parameters

Single-parameter optimization was carried out to optimize the process details. Three independent sets of experiments were done before concluding the results in all the studies, and the results are described based on the average values.

To optimize the incubation time, fermentation was carried out using 40 mL of semisynthetic medium in a 250-mL Erlenmeyer conical flask. One milliliter of spore suspension (6×10^7 spores) was used as the inoculum. The flasks were incubated for various time intervals (24–120 h). The biomass and the enzyme activities at various time points were calculated by removing the corresponding samples.

A master spore suspension of the fungal strain was prepared from PDA slants, and varying amounts of inoculum were used to study the effect of inoculum size on phytase production. SmF was carried out with 40 mL of semisynthetic medium inoculated with varying amounts (0.5, 1, 2, and 3 mL) of spore suspension. Fermentation was carried out at 45°C on a rotary shaker at 180 rpm for 72 h. Samples were removed after 72 h and assays were conducted.

To study phytase production in semisynthetic medium supplemented with either wheat bran extract or wheat bran, the semisynthetic medium was supplemented either with different percentages of wheat bran extract (5, 10, 20, 25, and 60% [v/v]) to make a 40-mL working volume or with different percentages of wheat bran particles (2.5, 3.75, and 5% [w/v]). The flasks were autoclaved and then inoculated with 1 mL of spore suspension and incubated at 45°C with shaking at 180 rpm for 72 h. Samples were withdrawn at desired time intervals and analyzed. Three independent sets of experiments were done before concluding the results.

To study the effect of supplementation of inorganic phosphate on phytase production, semisynthetic medium (without KH_2PO_4) supplemented with wheat bran (3.75% [w/v]) was used as the production medium, and it was further supplemented with different percentages of KH_2PO_4 (0.25–1% [v/w]). Fermentation was carried out for 72 h as mentioned earlier. Samples were analyzed for phytase activity.

To study the effect of surfactants, the production medium (semisynthetic plus 3.75% [w/v] wheat bran) was supplemented individually with different percentages of various surfactants such as Triton X-100, Tween-20, Tween-40, and Tween-80. The flasks were autoclaved and inoculated with 1 mL of spore suspension. The flasks were then incubated in a rotary shaker (150 rpm) at 45°C for 72 h. Samples were withdrawn periodically and used for enzyme assay.

To examine the effect of supplementation of nitrogen sources on phytase production, semisynthetic medium supplemented with wheat bran was prepared excluding peptone and used as the control. The medium was

supplemented with 1.8% (w/v) of five different nitrogen sources individually: peptone, ammonium sulfate, ammonium chloride, sodium nitrate, and urea. Fermentation was carried out as described earlier. After 72 h of fermentation, the samples were withdrawn and phytase activity was compared.

To study the effect of temperature on phytase activity, phytase assay was carried out using one of the best enzyme extracts at three different temperatures (37, 55, and 70°C), and changes in the activity level were noted.

Results and Discussion

The time course of phytase production and fungal growth (as dry weight) in SmF using the chemically defined semisynthetic medium is shown in Fig. 1. Enzyme production was directly associated with fungal biomass. Maximum enzyme activity (35.32 U/mL) was obtained after 72 h of fermentation, and fungal biomass (8.85 mg dry wt/mL of culture) was also at a maximum at this point. After 72 h, the culture attained stationary phase.

Wheat bran is a very common agroindustrial residue widely used for the purpose of enzyme production by filamentous fungi in solid-state fermentation *(17)*. It contains most of the vital constituents such as carbon, nitrogen, and amino acid sources required by the fungi. Hence, we decided to enrich the semisynthetic medium by adding either the wheat bran extract or the wheat bran particles themselves to enhance enzyme yield. Figure 2A shows the enzyme production in semisynthetic medium supplemented with wheat bran extract. It was observed that supplementation of wheat bran extract at a lower concentration (up to 40% [v/v]) did not enhance enzyme production. However, beyond 40% (v/v), the enzyme yield started to increase, and with 60% (v/v) supplementation a maximum of 68.75 U/mL was obtained, almost twice that of using semisynthetic medium alone. Supplementation beyond 60% (v/v) was not possible because then the medium turned into a semisolid owing to high viscosity. One possible reason for the not so encouraging results at lower concentrations might be the fact that in the wheat bran extract some of the vital factor such as phytic acid content might be less, which is why only with a high amount of extract supplementation did the enzyme yield start to increase. We therefore decided to use wheat bran particles to supplement the medium. The results are summarized in Fig. 2B. By adding 3.75% (w/v) wheat bran particles to the semisynthetic medium, the phytase yield was considerably increased and reached a maximum of 152.36 U/mL after 72 h of fermentation. By increasing the wheat bran proportion to 5% (w/v), the yield was reduced to 104.26 U/mL. Further supplementation was not possible, however, because the medium became semisolid and was not suited for SmF. The studies clearly demonstrate that wheat bran particles contain the stimulating factors, which may be missed while making the extract. Thus, by

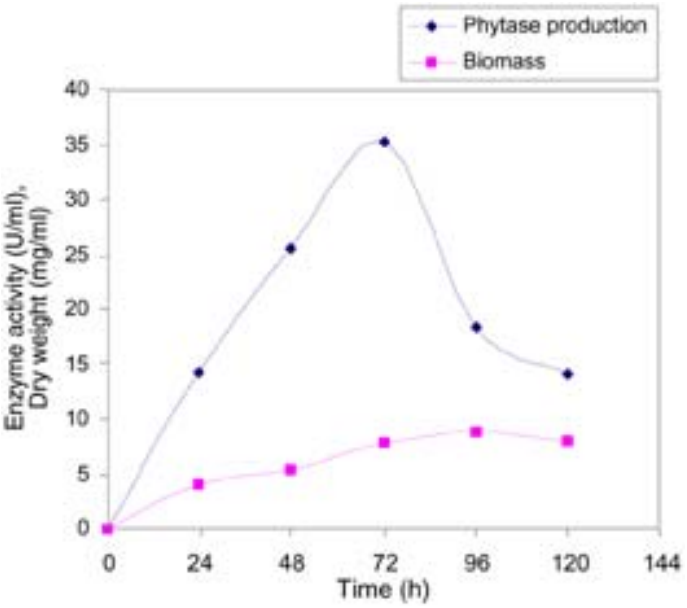

Fig. 1. Phytase production and growth in semisynthetic medium by *T. aurantiacus*.

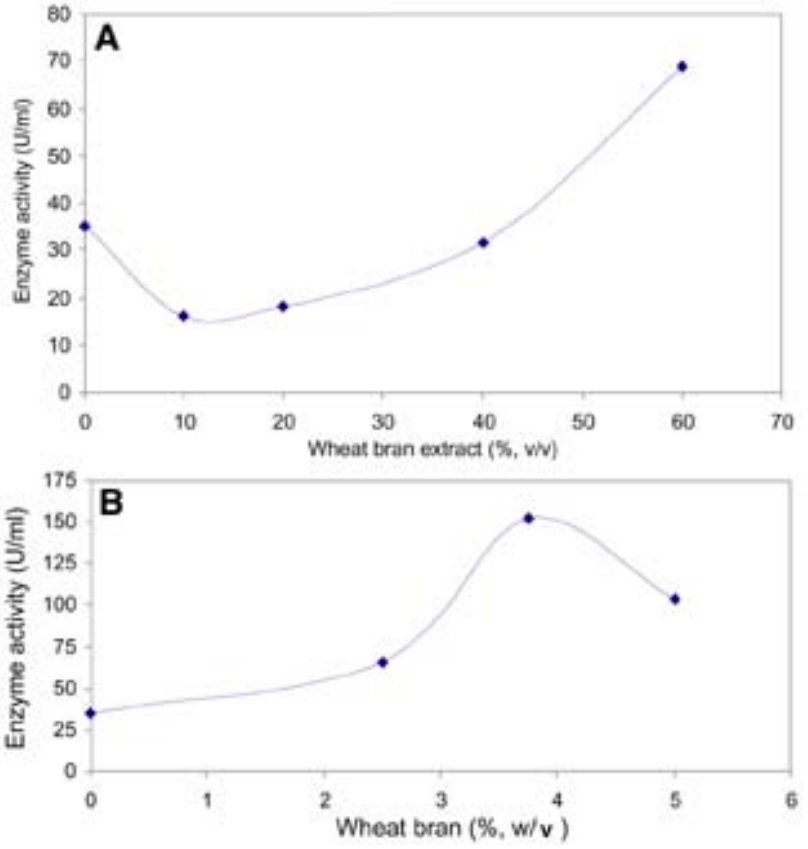

Fig. 2. Phytase production in semisynthetic medium supplemented with **(A)** wheat bran extract (72 h) and **(B)** wheat bran particles (72 h).

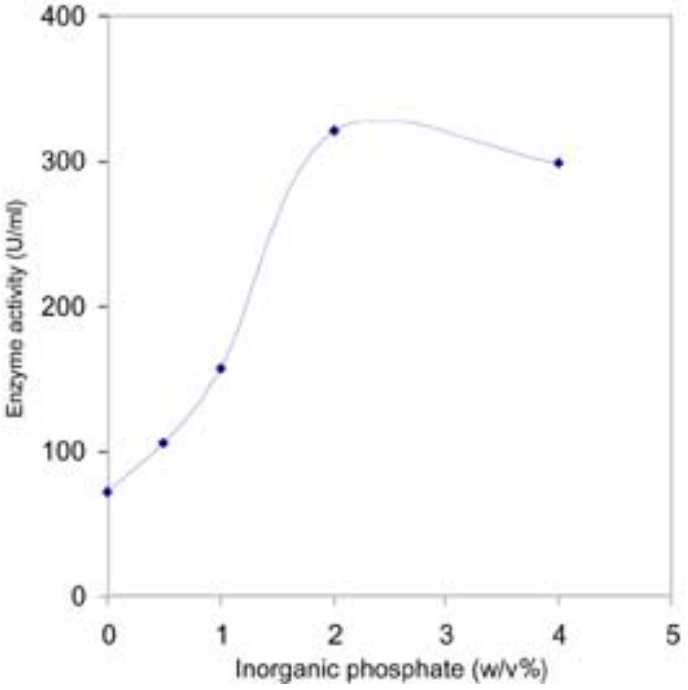

Fig. 3. Effect of inorganic phosphate on phytase production by *T. aurantiacus*.

using the basic semisynthetic medium supplemented with wheat bran particles, the enzyme activity was increased 4 to 4.5 times.

The inoculum size often determines the total biomass, substrate conversion, and product formation in any fermentation system. To determine the optimal inoculum size for phytase production, different concentrations of inoculum (0.5–3 mL) were used. One milliliter of the inoculum contained 6×10^7 spores of *T. aurantiacus*. Maximum enzyme activity (156 U/mL) was obtained when an inoculum of 1 mL of spore suspension was used (complete data not shown). The data indicated that higher or very low inoculum size was undesirable for phytase production.

To study the effect of phosphate concentration on enzyme production, semisynthetic medium was supplemented with KH_2PO_4 at various levels (0–4% [w/v]); the results are presented in Fig. 3. Interestingly, in the complete absence of free phosphate in the medium, phytase activity after 72 h was 72.34 U/mL. However, the yield proportionately increased with the initial phosphate concentration and reached a maximum of 321.32 U/mL in the presence of 2% (w/v) (0.8 g/40 mL of medium) free phosphate, and subsequently the activity declined at higher concentrations. Thus, it is assumed that free phosphate can stimulate phytase activity to a certain concentration level and it might be an inducer for the enzyme. There are similar reports in which supplementation of inorganic phosphorus at a certain level promoted phytase production *(18)*. However, there are also

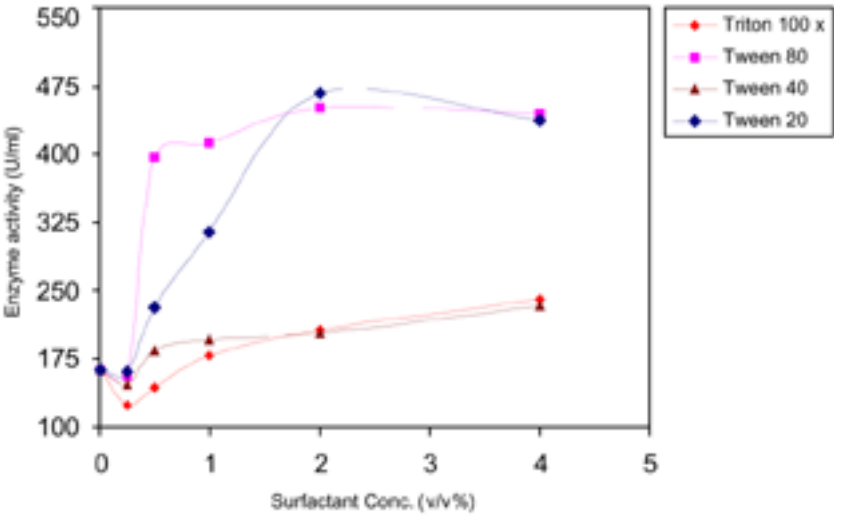

Fig. 4. Effect of surfactants on phytase production by *T. aurantiacus*.

reports stating that increased levels of inorganic phosphate in the medium suppressed synthesis of the enzyme *(19)*.

Surfactants have been reported to have an effect on the growth rate and enzyme production of fungi. In general, they have been used in biotechnology for improving the yields of a number of enzymes produced by fermentation *(20)*. However, the effects varied from organism to organism and from enzyme to enzyme. In an experiment to determine the effect of various surfactants (Triton X-100, Tween-80, Tween-40, and Tween-20) on enzyme production, the individual surfactants were added separately at various concentrations (0–4% [v/v]) to the production medium (semisynthetic medium supplemented with wheat bran particles) used for SmF. The results in terms of phytase activity are shown in Fig. 4. Among the four surfactants, Tween-80 and Tween-20 stimulated the enzyme production considerably. In the presence of 2% (v/v) Tween-80, the enzyme yield was 451.34 U/mL, and for Tween-20 it was 468.22 U/mL at the same concentration. It is also significant to note that the effect of Tween-80 was much more visible even at a lower concentration (396 U/mL with 0.5% [v/v]) than that of Tween-20 (231.8 U/mL with 0.5% [v/v]). The mechanisms by which surfactants enhance extracellular enzyme production were reported to be increased cell membrane permeability and change in lipid metabolism, which resulted in a higher release of these enzymes *(21)*. Like the results we obtained, El-Batal and Karem *(22)* reported that by using Tween-80, the highest level of phytase was obtained, and Triton X-100 had the maximum negative effect.

Studies on the impact of nitrogen sources (Fig. 5) revealed that instead of peptone, inorganic nitrogen sources such as ammonium sulfate and sodium nitrate could also be used as the major nitrogen source for phytase

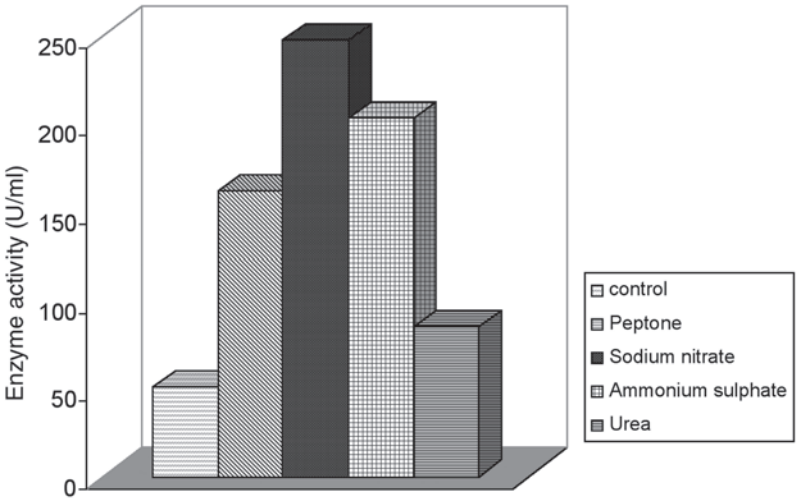

Fig. 5. Effect of nitrogen sources on phytase production by *T. aurantiacus*.

Table 1
Comparison of Thermostability of Phytase Enzyme
From *A. ficcum* (Mesophilic) and *T. aurantiacus* (Thermophilic)

Temperature (°C)	*A. ficcum* phytase activity (U/mL)	*T. aurantiacus* phytase activity (U/mL)
37	138.85	115.67
55	172.52	156.57
70	54.35	126.31

production. In fact, the yield was maximum (247.2 U/mL) with $NaNO_3$. Table 1 compares the temperature stability of two different microbial phytases obtained from a mesophilic culture (*A. ficcum*) and a thermophilic culture (*T. aurantiacus*). The enzyme activity was checked at 37, 55, and 70°C. In both cases, the temperature optimum was 55°C (172.52 U/mL for mesophilic culture and 156.57 U/mL for thermophilic culture). This is very similar to other fungal phytases that we have studied *(23)*. Interestingly, in the case of mesophilic phytase, the activity drastically decreased when the temperature was above 55°C. On the other hand, even at 70°C the phytase from *T. aurantiacus* had an activity of 126.31 U/mL, which is more than 75% of the activity obtained at 55°C. Thermostability is considered an important and useful criterion for industrial application of phytase. Naturally occurring phytase having the required level of thermostability for the application of animal feeding is very rare. The thermostability of this enzyme suggests, in addition to the use as feed enzyme, its potential biotechnological application in the pulp and paper industry as a novel biologic agent to remove phytic acid.

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