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Phytase Production by Thermophilic Mold *Sporotrichum thermophile* in Solid-State Fermentation and Its Application in Dephytinization of Sesame Oil Cake

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Abstract

The phytase production by *Sporotrichum thermophile* TLR50 was recorded on all the commonly used animal feed ingredients tested to varying degrees in solid-state fermentation. Enzyme production increased to 180 U/g of dry moldy residue (DMR) in sesame oil cake at 120 h and 45°C at the initial substrate-to-moisture ratio of 1:2.5 and a_w of 0.95. Supplementation of sesame oil cake with glucose and ammonium sulfate further enhanced phytase titer (282 U/g of DMR). An overall 76% enhancement in phytase production was achieved owing to optimization. The mold secreted acid phosphatase, amylase, xylanase, and lipase along with phytase. By the action of phytase, inorganic phosphate was liberated efficiently, leading to dephytinization of sesame oil cake.

Index Entries: Phytase; thermophilic mold; *Sporotrichum thermophile* TLR50; sesame oil cake; solid-state fermentation; dephytinization.

Introduction

Phytase (*myo*-inositol hexakisphosphate 3-phosphorylase, EC 3.1.3.8) is an important feed additive to increase the availability of organic phosphate and other nutritionally important minerals for monogastrics by the hydrolysis of phytic acid (*myo*-inositol hexakis dihydrogen phosphate), an antinutritional factor present in most of the cereal- and legume-based diets

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(1,2). Because phytin is not metabolized in the intestinal tract of monogastric animals that consume rations containing cereals and legumes, much of the phosphate passes through the animal urine and feces and pollution is increased in aquatic bodies *(3)*. The commercially available phytase is produced by *Aspergillus ficuum* and *Aspergillus niger* strains by submerged fermentation (SmF). The cost of commercial phytase supplementation is about $2 to $3/metric t of feed *(4)*. A more economical alternative method for the production of the enzyme would be solid-state fermentation (SSF) *(5)*.

SSF is generally defined as the growth of microorganisms on solid substrate in the absence or near absence of free-flowing water. Filamentous fungi have been employed for the production of phytases by SSF *(1,6–8)*. Solid substrates supply all the nutrients to the microbial culture and serve as an anchorage for the cells in SSF. For each fungal species, a suitable substrate has to be selected for the growth and enzyme production. Attention is being focused on the utilization of agricultural crops and their residues *(7)*. Oilseed cakes are commonly used in animal feeds, because they are a rich source of protein and energy for ruminants. India is one of the world's leading oilseed producers, where the total production is more than 25 million t per annum and the exports account for more than 4.3 million t of oil meals, valued at US$800 million *(8)*. Oil cakes are economically cheaper and possess a higher protein content (15–50%) than other forms of agricultural residues. Sesame oil cake is a valuable poultry feed ingredient, because its protein is rich in methionine, cystine, arginine, and tryptophan. It, however, contains a high level of phytic acid, an antinutrient that reduces its suitability for use as animal feed *(8)*.

Phytase may be produced directly in SSF by filamentous fungi on the selected animal feeds, and the crude product may be mixed in feed rations as a value-added supplement *(9)*. The fungal product contains not only phytase, but also accessory enzymes, fungal proteins, and organic acids that increase feed digestibility and access to phytate in plant cells *(1,10)*.

In the present investigation, phytase production by four thermophilic fungal strains—*Sporotrichum thermophile* A64, *S. thermophile* TLR50, *Humicola lanuginosa* DCC, and *H. lanuginosa* PT102—was compared in SSF on two feed ingredients (wheat bran and sesame oil cake) that are commonly used in animal feeds. The production of phytase by *S. thermophile* TLR50 in SSF and its applicability in liberating inorganic phosphate from sesame oil cake by dephytinization were investigated.

Materials and Methods

Fungi and Preparation of Inoculum

The thermophilic molds *S. thermophile* A64, *S. thermophile* TLR50, and *H. lanuginosa* PT102 were isolated from soil samples collected from Gir forest (Gujarat), Tilyar lake in Rohtak (Haryana), and Patiala (Punjab), India, respectively. *H. lanuginosa* DCC was obtained from the culture collection of the Department of Microbiology, University of Delhi South Cam-

pus, New Delhi. The molds were grown and maintained on Emerson YpSs agar *(11)* slopes. The conidiospores from 6-d-old fully sporulated solid media slopes were harvested by washing with normal saline containing 0.1% Tween-80, and the spore suspension was adjusted to 10^8 colony-forming units (CFU)/mL for inoculation.

Phytase Production

All the substrates (wheat bran, wheat straw, cotton oil cake, mustard oil cake, sesame oil cake, corncob, sugarcane bagasse, rice straw) used in SSF were obtained locally. Ten grams of air-dried substrate was placed in 250-mL Erlenmeyer flasks and moistened with 25 mL of distilled water. The wet substrate was sterilized at 121°C for 20 min. After cooling, the substrate was inoculated with 1 mL of spore suspension (10^8 CFU/mL). The flasks were incubated for 4 d at 45 ± 1°C in a humidified chamber at 70% relative humidity.

For comparison, phytase production was also carried out in 250-mL Erlenmeyer flasks containing 50 mL of starch-glucose-peptone broth (3.0 g/L of starch, 1.0 g/L of glucose, 2.0 g/L of peptone, 3.0 g/L of sodium phytate, 0.01 g/L of $FeSO_4$, 0.01 g/L of $MnSO_4$, 0.2 g/L of $CaCl_2$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 10.0 [v/v] g/L of Tween-80, pH 5.0). The flasks were inoculated with spore suspension (1×10^7 CFU/50 mL of medium) and incubated for 72 h at 45°C and 250 rpm in an incubator shaker.

Optimization of Process Parameters

SSF was carried out to optimize the fermentation process parameters, which included suitable substrate, incubation time, incubation temperature, initial moisture content of the substrate, and water activity (a_w). These parameters influenced phytase production significantly. The effect of water activity on phytase production was studied using glycerol as a depressant of water activity *(12)*. The solid medium was supplemented with additional carbon (glucose, sucrose, maltose, fructose, starch; all at 2% [w/w]) and nitrogen (yeast extract, beef extract, ammonium nitrate, ammonium sulfate, sodium nitrate; all at 1% [w/w]) sources. The effect of different concentrations of glucose (1–8% [w/w]), ammonium sulfate (0.5–4% [w/w]), and phytic acid (0.1–1% [w/w]) on phytase production was also assessed. For the optimization of various process parameters influencing phytase production, a parameter was varied by keeping all the others constant, and this was incorporated at its optimum level while optimizing the next parameter.

SSF in Trays

Enamel-coated metallic trays measuring 28 × 24 × 4 cm and 40 × 30 × 5 cm containing 50 g/100 g and 200 g of substrate, respectively, were moistened with distilled water (1:2.0 [w/v]). Filter-sterilized glucose and ammonium sulfate were added to the medium after autoclaving in such a way

that the final moisture ratio was 1:2.5. The inoculum was mixed with a sterile spatula aseptically, and the trays were incubated at 45°C for 5 d in a humidified chamber.

Measurement of Phytic Acid

Phytic acid reduction from sesame oil cake during SSF was determined in the solid substrate by extracting with 0.2 *N* HCl with constant shaking at 200 rpm for 1 h. The suspension was centrifuged at 8000*g* for 15 min, and the supernatant was used for estimating phytic acid according to Haug and Lantzsch *(13)*. To 0.5 mL of sample, 1 mL of ferric ammonium sulfate solution was added in a covered tube and kept in a boiling water bath for 30 min. After cooling in ice water for 15 min, 2 mL of bipyridine solution was added once the tubes reached room temperature, and the absorbance was measured at 519 nm.

Profile of Hydrolytic Enzymes Secreted by Thermophilic Mold S. thermophile *Under Optimized Conditions*

The profile of hydrolytic enzymes secreted by the thermophilic mold in SSF was studied under optimized conditions. Ten grams of sesame oil cake were placed in 250-mL Erlenmeyer flasks and moistened with distilled water (moisture ratio of 1:2). Filter-sterilized glucose and ammonium sulfate were added to the medium after autoclaving in such a way that the final moisture ratio was 1:2.5. The flasks were inoculated with 1 mL of spore suspension (1×10^8 CFU/mL) and incubated at 45°C for 15 d in a humidified chamber. A set of three flasks was harvested after regular intervals, and extracts were used in enzyme assays.

Dephytinization of Sesame Oil Cake by Action of Phytase *of* S. thermophile *TLR50*

Five grams of washed and dried sesame oil cake was mixed with 50 mL of 0.1 *M* sodium acetate buffer (pH 5.0) and incubated at 50°C and 100 rpm with 1 mL of enzyme sample (10 U). One milliliter of sample was withdrawn at the desired intervals and used for estimating inorganic phosphate liberated according to Fiske and Subbarow *(14)*. All experiments were carried out in triplicate and the average values are presented.

Analytical Methods and Enzyme Assays

The fermented solid substrates were extracted with distilled water containing 0.1% Tween-80 (20 mL of water/g of dry substrate) while shaking for 1 h at 200 rpm. The suspension was centrifuged at 10,000*g* for 10 min, and the supernatant was used as the source of enzyme(s).

Phytase was assayed by measuring the liberation of inorganic phosphate from sodium phytate *(15)* at pH 5.0 and 60°C. The liberated inorganic phosphate was estimated according to the method of Fiske and Subbarow

(14). One unit of phytase is defined as the amount of enzyme that releases 1 nmol of inorganic phosphorus per second under the assay conditions.

Amylase present in the enzyme extracts was assayed according to Kumar and Satyanarayana *(16)* at pH 5.0 and 60°C. The liberated reducing sugars were determined using dinitrosalicylic acid (DNS) *(17)*. One unit of amylase is defined as the amount of enzyme that liberates 1 nmol of reducing sugar as glucose equivalent per second under the assay conditions.

Acid phosphatase activity was measured according to Bartlett and Lewis *(18)* at pH 5.0 and 60°C. One unit of phosphatase is defined as the amount of enzyme that liberates 1 nmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per second under the assay conditions.

Xylanase was assayed according to Archana and Satyanarayana *(19)* at pH 5.0 and 60°C. The liberated reducing sugars were determined using DNS *(17)*. One unit of xylanase is defined as the amount of enzyme that liberates 1 nmol of reducing sugar as xylose per second under the assay conditions.

Lipase was assayed by estimating the liberation of *p*-nitrophenol from *p*-nitrophenyl palmitate *(20)* at pH 7.0 and 60°C. One unit of lipase is defined as the amount of enzyme that liberates 1 nmol of *p*-nitrophenol per second under the assay conditions. The production of enzymes is expressed as units per gram of dry moldy residue (DMR).

Results and Discussion

Selection of a Potent Thermophilic Mold and Substrate for Phytase Production

Initially four thermophilic fungal strains—*S. thermophile* TLR50, *S. thermophile* A64, *H. lanuginosa* PT102, and *H. lanuginosa* DCC—were screened for phytase production on sesame oil cake and wheat bran in SSF. Of these molds, phytase secretion by *S. thermophile* TLR50 was significantly greater on both wheat bran (66.68 ± 3.46) and sesame oil cake (148.25 ± 8.78), but 2.5 times greater on sesame oil cake than on wheat bran. Comparisons of phytase production using other agroresidues, shown in Fig. 1, indicated that sesame oil cake supported higher phytase titers than mustard oil cake and wheat bran. Bogar et al. *(21)* reported secretion of phytase by *Mucor racemosus* NRRL 1994 on sesame oil cake, but at a lower specific productivity than that on coconut oil cake in SSF. A high titer of phytase was attained on a mixture of coconut and sesame oil cake in *Rhizopus oryzae* NRRL 1891 *(8)*, and *A. niger* NCIM563 produced a higher phytase titer on cowpea meal than wheat bran *(22)*. *Rhizomucor pusillus (23)* and *A. ficuum* NRRL 3135 *(9)* secreted phytase optimally on wheat bran. It is evident from these observations that fungal strains have a varied preference for substrates for phytase production.

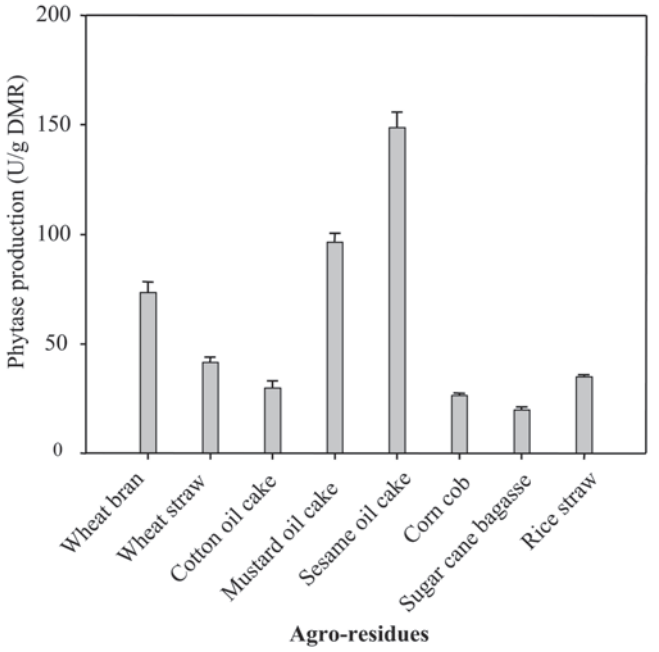

Fig. 1. Phytase production by *S. thermophile* TLR50 in various agroresidues in SSF.

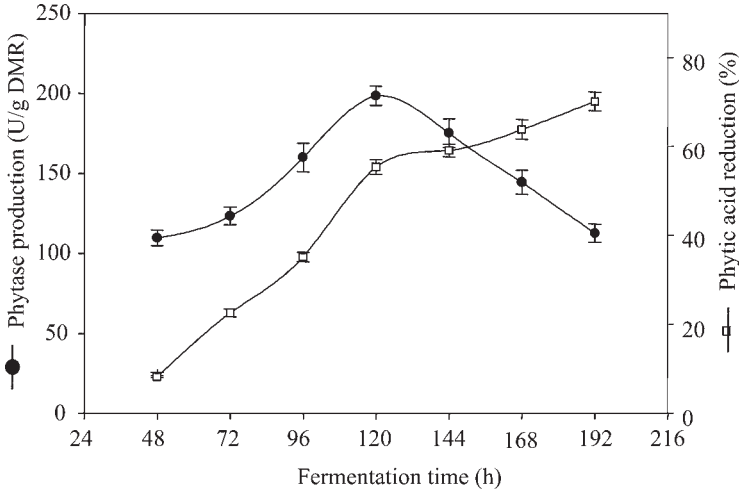

Fig. 2. Effect of fermentation time on phytase production by *S. thermophile* TLR50 in SSF.

Parametric Optimization for Production of Phytase

Based on these observations, *S. thermophile* TLR50 was selected for a detailed investigation of the optimization of phytase secretion using sesame oil cake as solid substrate. Phytase production by *S. thermophile* increased from inoculation through 120 h of incubation and declined thereafter (Fig. 2),

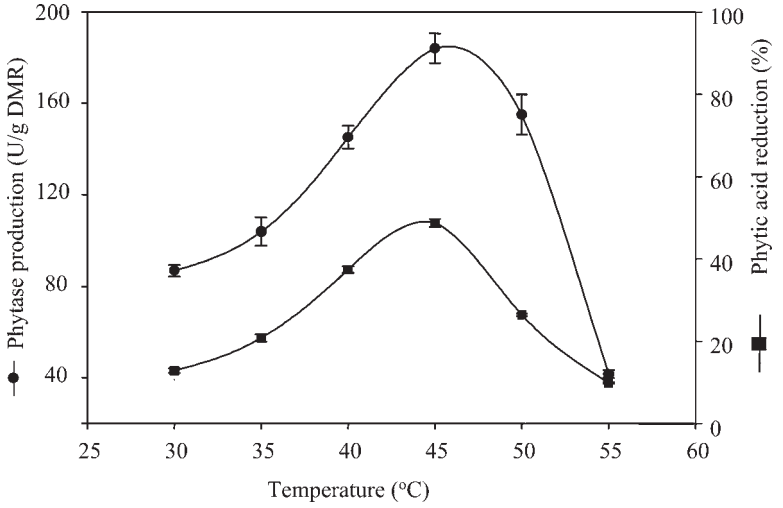

Fig. 3. Effect of temperature on phytase production in SSF.

while reducing phytic acid content of sesame oil cake to 50% during this fermentation time and up to 70% after 192 h. The decline in enzyme synthesis after 120 h could be owing to the reduced nutrient levels in solid substrate and denaturation of the enzyme *(24)*. A peak in phytase production was achieved after 72 and 96 h in *A. ficuum* and *R. oligosporus (24)*, respectively. In *A. niger* NCIM563, high phytase titer was, however, attained in 168 h with concomitant reduction in phytic acid *(22)*.

Incubation temperature influenced the rate of phytase production by *S. thermophile* (Fig. 3). Maximum phytase production was achieved at 45°C, which is the optimum temperature for the growth of this mold. With further increase in temperature, there was a sharp decline in enzyme titers. *R. pusillus (23)* and *Aspergillus* spp. *(3,6,25)* secreted phytase optimally at 50 and 30°C, respectively, which were their temperature optima for growth.

Initial moisture content is a critical factor that influences microbial growth and enzyme production. Moisture is a factor that is intimately related to the definition of SSF because it is necessary for new cell synthesis *(26)*. The optimum level of initial moisture content in solid support required for enzyme production during SSF was determined by altering the volume of moistening agent added to the solids so that different moisture levels were attained. In this investigation, the highest enzyme production was recorded at a substrate-to-moisture ratio of 1:2.5 (Fig. 4) accompanied by a reduction in phytic acid content of sesame oil cake. Any further increase in the initial moisture content decreased enzyme production. With increasing water content and constant substrate volume, the air content of the substrate decreases owing to the flooding of interparticle space of the substrate that results in reduced growth and proliferation of fungal mycelia *(8)*. Water contents on either side of this moisture level were found to decrease the decomposition rate of the total organic matter *(5)*. *R. pusillus* produced high

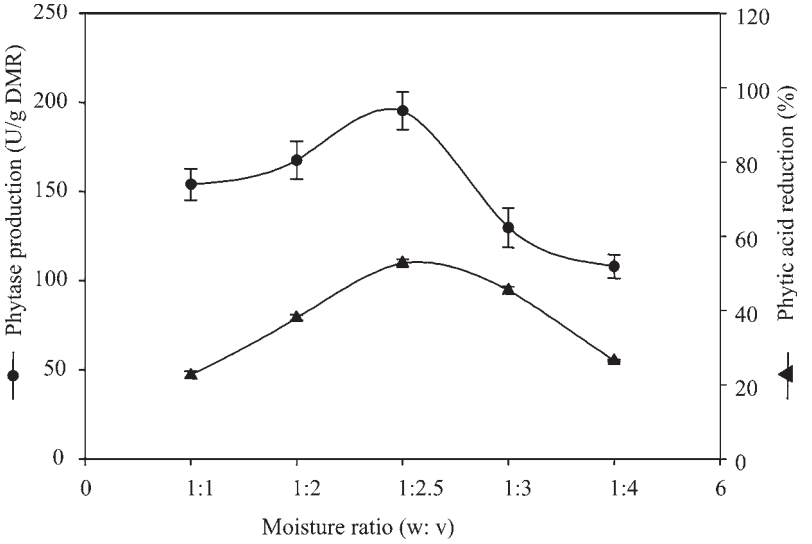

Fig. 4. Effect of substrate:moisture ratio on production of phytase.

phytase titers in SSF at a substrate-to-moisture ratio of 1:2 *(23)*, whereas a ratio of 1:1 was needed for *A. niger* NCIM563 *(22)* and *A. ficuum (6)*.

The effect of water activity (a_w) on phytase production in SSF was assessed by adding glycerol as a depressant of a_w. *S. thermophile* TLR50 secreted maximum phytase titers at a_w 0.95 (182.56 U/g of DMR), there was a drastic decline in phytase production below this value, and beyond 0.85, there was no growth or phytase production. Pandey et al. *(27)* and Kumar and Satyanarayana *(16)* also observed glucoamylase production at a_w 0.93 and 0.95, respectively. The a_w of the substrate is an important factor in SSF, because at relatively low moisture content, growth and metabolism can be limited. In SSF, the a_w of the substrate influenced the enzyme yield and protein stability as well *(28)*.

Effect of Supplementation of Sesame Oil Cake With Carbon and Nitrogen Sources

For the initiation of growth and metabolism, fungi require carbon sources in easily available form *(24)*. Among all the carbon sources tested, glucose supplementation resulted in a high phytase production (256.78 U/g of DMR) (Fig. 5), but only by an approx 10% difference compared to other additives and 25% compared to the control without additional carbohydrate. This was similar to that observed in *M. racemosus* NRRL 1994 *(21)* and *R. oryzae (8)*. Starch was found to be a suitable supplement for phytase production by *A. ficuum* NRRL 3135 *(9)*.

Phytase production was enhanced when solid substrate was supplemented with inorganic nitrogen sources. Ammonium sulfate at 1% supplementation supported the highest enzyme production by *S. thermophile* TLR50 (Fig. 6) as reported in *M. racemosus* NRRL 1994 *(21)*. Ammonium

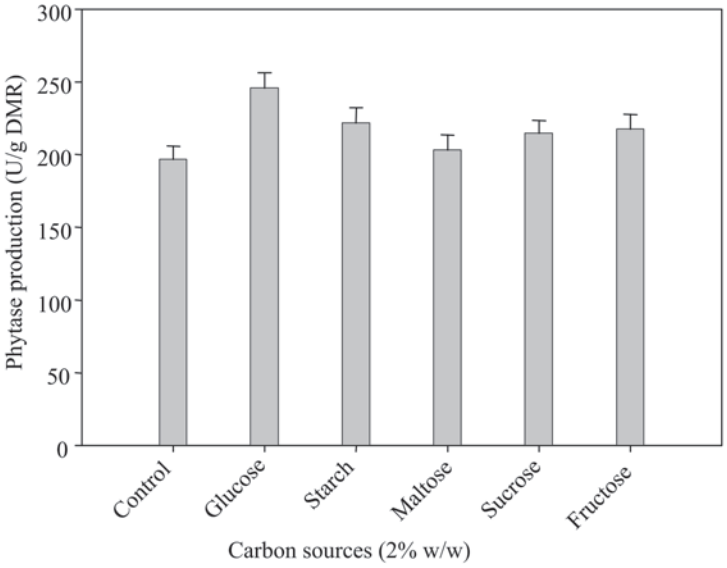

Fig. 5. Effect of supplementation of sesame oil cake with different carbon sources on phytase production in SSF.

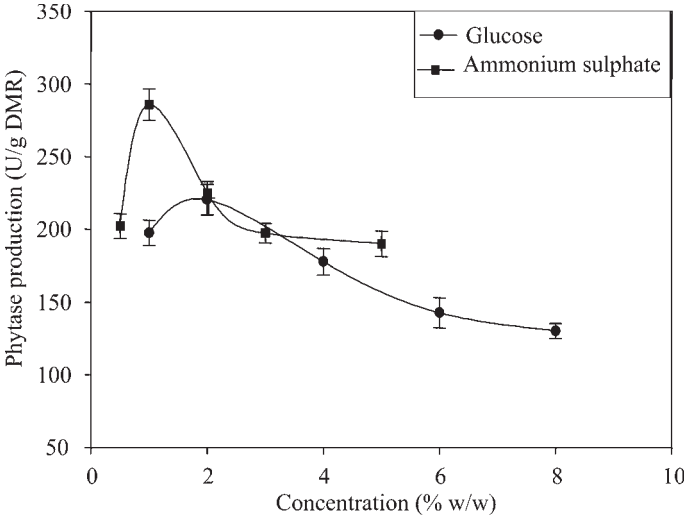

Fig. 6. Effect of different concentrations of glucose and ammonium sulfate on phytase production in SSF.

nitrate caused a high phytase secretion in *A. ficuum* NRRL 3135 *(9)*, *R. oryzae (8)*, and *A. niger* PD *(3)*. The effect of nitrogen sources on enzyme production by fungi is complicated. It is generally accepted that inorganic nitrogen sources are more easily assimilated than organic nitrogen sources by fungi *(3)*. By contrast, asparagine and corn steep liquor supported a high phytase titer in *R. pusillus (23)*.

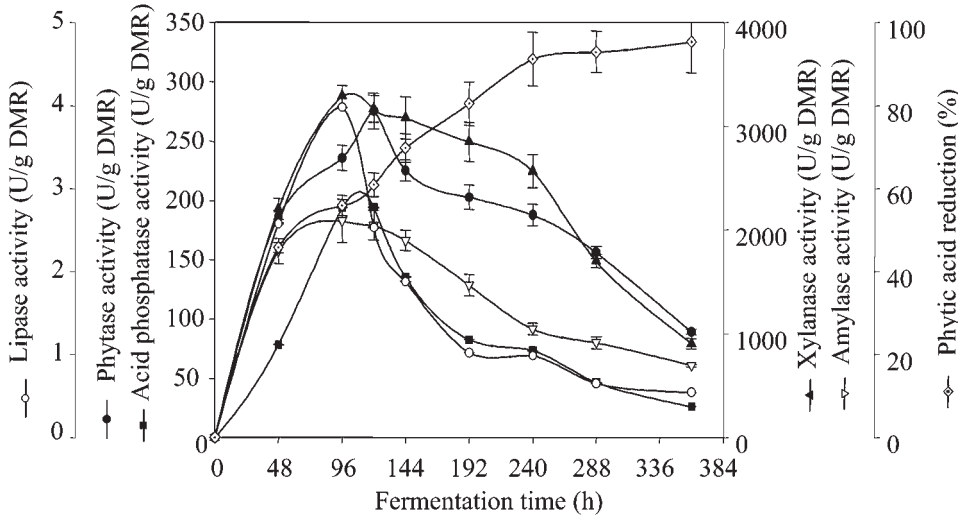

Fig. 7. Profile of hydrolytic enzymes secreted by *S. thermophile* under optimized conditions in SSF.

Glucose at 2% (w/w) supported a high phytase production (Fig. 6), as recorded by Ebune et al. *(6)*, whereas supplementation of glucose at 5.2% enhanced phytase production by *A. ficuum* NRRL 3135 in canola meal with a concomitant reduction in phytic acid. Similarly, in *R. oryzae (8)* and *M. racemosus* NRRL 1994 *(21)*, the addition of glucose at 1.0 and 4.51% supported phytase production, respectively. Phytase production was not affected by the supplementation of phytic acid to the solid substrate (data not shown) as observed in *R. oligosporus (7)*.

Comparison of Phytase Production in SMF and SSF

Phytase production by *S. thermophile* was 22-fold higher in SSF (276 U/g of DMR) than in SmF (12.60 U/mL), based on the enzyme titer per gram of DMR in SSF to per milliliter in SmF *(16)*. Similar observations were made earlier for glucoamylase and α-amylase production by *Thermomucor indicae-seudaticae (16)* and *Bacillus coagulans (29)*, respectively. This marked increase in enzyme production in SSF has been attributed to intimate contact of the organism with the substrate and minimization of catabolite repression owing to lack of uniform distribution of the catabolite.

Other Hydrolytic Enzymes Secreted by S. thermophile and Dephytinization of Sesame Oil Cake

S. thermophile TLR50 secreted acid phosphatase, xylanase, lipase, and amylase along with phytase in SSF (Fig. 7) as recorded in *A. ficuum* NRRL 3135 *(9)* and *M. racemosus* NRRL 1994 *(21)*. Kaur and Satyanarayana *(30)* recently reported the secretion of cellulase, xylanase, and pectinase by the thermophilic mold *Sporotrichum thermophile* Apinis in SSF. Phytic acid

present in sesame oil cake was completely degraded by *S. thermophile* in 288 h, which is slower than that by *A. niger* NCIM 563, wherein total reduction in phytic acid content of wheat bran was achieved in 188 h *(22)*.

The phytic acid of the sesame oil cake was also efficiently hydrolyzed by phytase of *S. thermophile,* leading to the liberation of inorganic phosphate and dephytinization of sesame oil cake. There was a gradual increase in the liberation of inorganic phosphate up to 60 h, followed by stabilization and decline, which could be owing to the denaturation of the enzyme and/or end-product inhibition.

SSF in Enamel Trays

The enzyme yield in trays (260–255 U/g of DMR) was comparable with that in Erlenmeyer flasks (276 U/g of DMR) as reported for the production of phytase in *A. niger* NCIM 563 *(22)* and α-amylase in *B. coagulans (29)*. This suggested a possibility of scale-up of phytase production.

The enzyme produced in solid substrate can be easily mixed with other ingredients in a feed ration in contrast to the SmF product, which is highly diluted. The economy with the enzyme produced in SSF appeared to be favorable, as reported by Bogar et al. *(9,21)*. Sesame oil cake supported high phytase production by the thermophilic mold *S. thermophile* TLR50 in SSF. A 76% enhancement in phytase production was achieved owing to optimization. The production of phytase was 22-fold higher in SSF than in SmF. The observations that sesame oil cake supports luxuriant fungal growth, phytase production, and efficient liberation of inorganic phosphate by the action of the enzyme with the concomitant reduction in phytate content, as well as its acid and thermal stability, appear to be of commercial significance.

Acknowledgment

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