0273-2289/02/102–103/0251/$12.50

Solid-State Fermentation for Production of Phytase by *Rhizopus oligosporus*

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Abstract

Solid-state fermentation of coconut oil cake has been carried out with *Rhizopus oligosporus* for the production of phytase. Phytase is used commercially in the animal feed industry to improve animal performance because there is a substantial and growing interest among swine and poultry producers in the application of phytase to improve the nutritional quality in animal feeds. Demonstrated benefits include improved feed yield ratios and reduction in the environmental costs associated with the disposal of animal wastes. We report the production of extracellular phytase by *R. oligosporus* under solid-state fermentation using coconut oil cake as substrate. Maximal enzyme production (14.29 U/g of dry substrate) occurred at pH 5.3, 30°C, and 54.5% moisture content after 96 h of incubation. The addition of extra nutrients to the substrate resulted in inhibition of product formation. The results indicate the scope for production of phytase using coconut oil cake as solid substrate without additional nutrients.

Index Entries: Phytase; *Rhizopus oligosporus*; coconut oil cake; solid-state fermentation.

Introduction

Phytase, or myoinositol hexakisphosphate phosphohydrolase (EC 3.1.3.8), is an important feed additive to increase the availability of phosphorus and

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other nutritionally important minerals for monogastric animals by the enzymatic hydrolysis of phytic acid (myoinositol hexakisphosphate), an antinutritional factor present in most of the cereals and legume-based feeds *(1)*. The principal end products of phytase action are phosphoric acid and myoinositol. Most cereals and legumes are rich in protein and fat, but the presence of phytic acid discourages their use in food. Phytic acid acts as an antinutrient owing to its chelation of various metals and binding of protein. This diminishes the bioavailability of proteins and nutritionally important minerals.

Solid-state fermentation is generally defined as the growth of microorganisms on (moist) solid substrates in the absence or near absence of free water *(2–5)*. Solid-state fermentation has several advantages over submerged fermentation, including higher product titers, lower wastewater output, reduced energy requirements, simplicity, absence of foam formation, simpler fermentation media, lesser fermentation space requirement, easier aeration, reduced bacterial contamination, high reproducibility, absence of rigorous control of fermentation parameter, less complex plant, and use of less water. In addition, since spores are used directly, the inoculum tank can be avoided *(2,3)*.

The selection of a substrate for solid-state fermentation process depends on several factors mainly related to cost and availability and thus may involve screening of several agroindustrial residues. In solid-state fermentation processes, the solid substrate supplies the nutrients to the microbial culture growing in it and also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganism growing in it should be considered an ideal substrate *(5)*.

Research on the selection of a suitable substrate has mainly centered on tropical agroindustrial crops and residues. These include crops such as cassava, soybean, sugar beat, sweet potato, potato, and sweet sorghum; crop residues such as bran and straw of wheat and rice, bagasse of cassava and sugarcane; residues of the coffee-processing industry such as coffee pulp, coffee husk; residues of fruit-processing industries such as pomace of apple and grape, pineapple waste, banana waste; and waste of oil-processing mills such as coconut oil cake, soybean cake, peanut cake, canola meal, and palm oil waste. Many processes have been developed that utilize these as raw material for the production of bulk chemicals and value-added fine products such as ethanol, single-cell protein, mushrooms, enzymes, organic acids, and biologically active secondary metabolites *(6–8)*.

Utilization of agricultural wastes and other cheap natural substrates is one among the many major advantages of solid-state fermentation because of its great economic viability. In this regard, the prime objective remains to recycle the less-valued byproduct into value-added products. In this context, the present study was undertaken to explore the possibility of utilizing coconut oil cake (copra cake), which is a byproduct obtained after oil extraction from dried coconut kernel, as a substrate for the production of phytase enzyme in solid-state fermentation.

Materials and Methods

Microorganism and Preparation of Inoculum

Rhizopus oligosporus obtained from the culture collection of the Technical University of Budapest, Hungary, was used. It was maintained by monthly subculture on potato dextrose agar (PDA) slants. Spore inoculum was prepared from freshly raised 7-d-old PDA (Himedia, India) slope culture. To fully sporulated agar slope culture, 10 mL of sterile distilled water with 0.1% Tween-80 was added by means of a sterile pipet. Then the spores were scrapped using an inoculation needle under strict aseptic conditions. The spore suspension obtained was used as the inoculum. Viable spores in the fully sporulated slants were determined by the plate-count (colony count) technique, using a colony counter.

Solid-State Fermentation

Ten grams of copra cake collected from the local market were placed in 500-mL Erlenmeyer flasks; moistened with 12 mL of mineral salt solution having a composition of 0.5% NH_4NO_3, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.1% NaCl; autoclaved for 20 min at 121°C; and cooled to room temperature. The sterilized solid substrate media were inoculated with 1 mL of the inoculum. The contents were mixed thoroughly and incubated at 30°C in an incubator. All the experiments were done in duplicate, and mean values are reported with an SD of ±3.1.

Optimization of Process Parameters

Optimization of various physicochemical parameters required for maximum phytase production by *R. oligosporus* under solid-state fermentation was evaluated. The various process parameters optimized for enhanced phytase production were incubation time (24–168 h), incubation temperature (25–60°C), initial pH of the mineral salt solution (3.0–8.0), initial moisture content of the medium (37.5–70.5%), inoculum size (0.5×10^6 to 5×10^6 spores), additional carbon sources (maltose, glucose, mannitol, sorbitol, lactose, and sucrose at 1% [w/v]), additional organic and inorganic nitrogen sources (peptone, yeast extract, malt extract, beef extract, ammonium sulfate, ammonium chloride, sodium nitrate, and potassium nitrate at 1% [w/v]), and effect of the addition of phytic acid (0.1–0.5% [w/v]).

Analytical Methods

Enzyme Extraction

From the fermented solids, enzyme extraction was carried out using distilled water with 0.1% (v/v) Tween-80. Extraction was done in two steps, each time with 44 mL of distilled water so that the final extraction volume was 100 mL (88 mL of distilled water + 12 mL of initial moistening medium). First, the fermented substrates were mixed with 44 mL of distilled water, and the flasks were kept on a rotary shaker at 200 rpm for 30 min. Second,

the solids were separated from the solution by filtering through cheesecloth. This step was repeated and the solution was centrifuged at 12,000*g* for 20 min at 4°C in a refrigerated centrifuge. The supernatant designated as crude enzyme was collected and used for enzyme assay.

Enzyme Assay

Phytase was assayed according to Harland and Harland *(9)*. One international unit of phytase was defined as the amount of enzyme required for releasing 1 µmol of inorganic phosphorus per minute at a given temperature and pH. Enzyme yield was expressed as U/gds (gram dry substrate).

Results and Discussion

Solid substrates employed in solid-state fermentation processes are insoluble in water and act as a source of carbon, nitrogen, and minerals as well as growth factors. Filamentous fungi are able to penetrate deep into the solid substrate particles for nutrient uptake, while bacteria and yeast grow by adhering to the surface of the solid substrate particles. Since solid-state fermentation processes using nutritionally rich substrates such as coconut oil cake, which we used in the present study, have immense potential, use of such materials as supports for solid-state fermentation has been recommended *(4)*. The major components in coconut oil cake include starch (18.82%), protein (14.34%), lipids (6.68%), and sugars (6.31%) *(10)*.

The procedure adopted for optimization of various process parameters influencing phytase production was to evaluate the effect of an individual parameter and to incorporate it at the optimal level in the experiment before optimizing the next parameter.

From the experiment to optimize the incubation time required for maximal phytase production by *R. oligosporus* in the coconut oil cake system, the results presented in Fig. 1 suggested that phytase production increased progressively along with an increase in incubation time until 96 h (14.29 U/g of dry substrate). However, the enzyme yield declined during further incubation. The decrease in enzyme yield after 96 h could be owing to the increased biomass production, which, in turn, might have resulted in the depletion of carbon source and nutrients in the medium, affecting the enzyme synthesis. It could also be the result of poisoning and denaturation of the enzyme caused by interaction with other components in the medium *(11)*.

Optimum pH of the solid substrate required for maximal phytase production during solid-state fermentation was evaluated using mineral salt solutions of various pH levels (3.0–8.0). The results obtained revealed almost the same enzyme yield for all the pHs tested. To find an explanation for this, the final pH of the solid substrates was noted after autoclaving. The results presented in Table 1 indicate that autoclaving changed the initial pH (ranging from 3.0 to 8.0) of the substrate to 5.3 ± 0.2. The change in substrate pH after autoclaving might have occurred because of the buffering capacity of the complex natural substrates. Most microbial extracel-

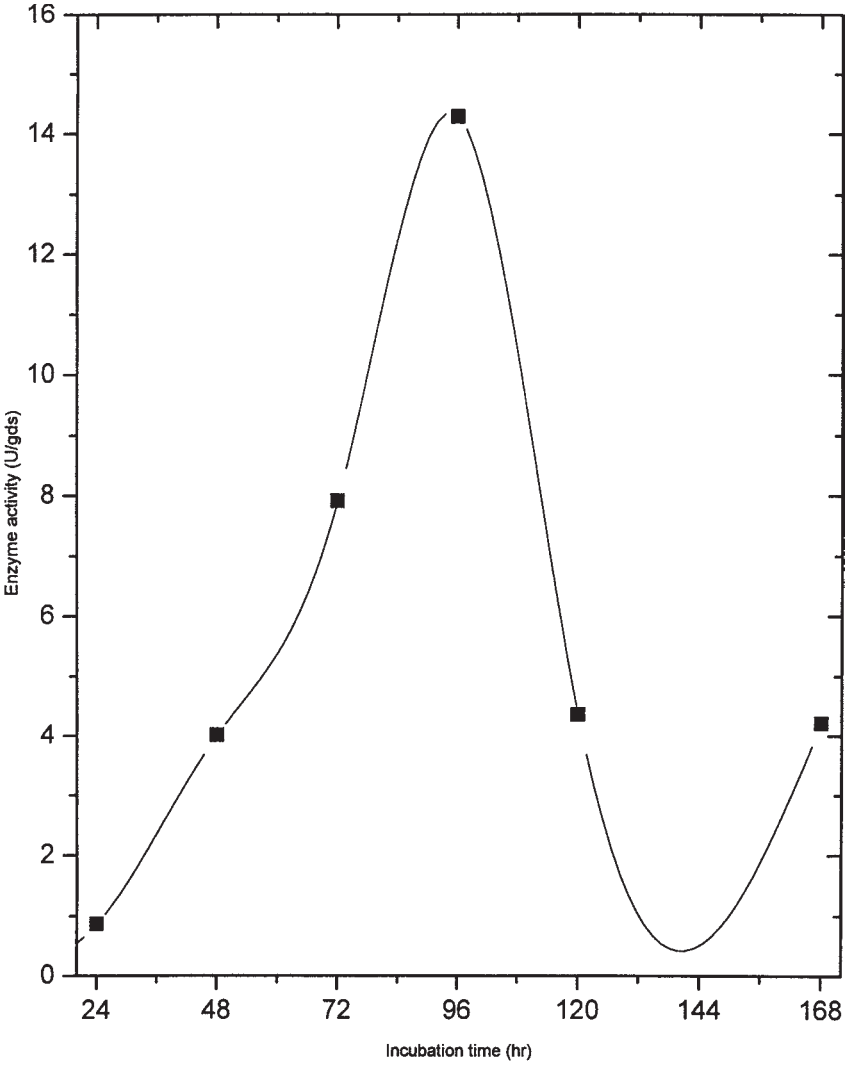

Fig. 1. Phytase production during various incubation times.

Table 1
pH of Media After Autoclaving

pH of mineral salt solution	Final pH
3.0	5.38
4.0	5.36
5.0	5.37
6.0	5.39
7.0	5.42
8.0	5.37

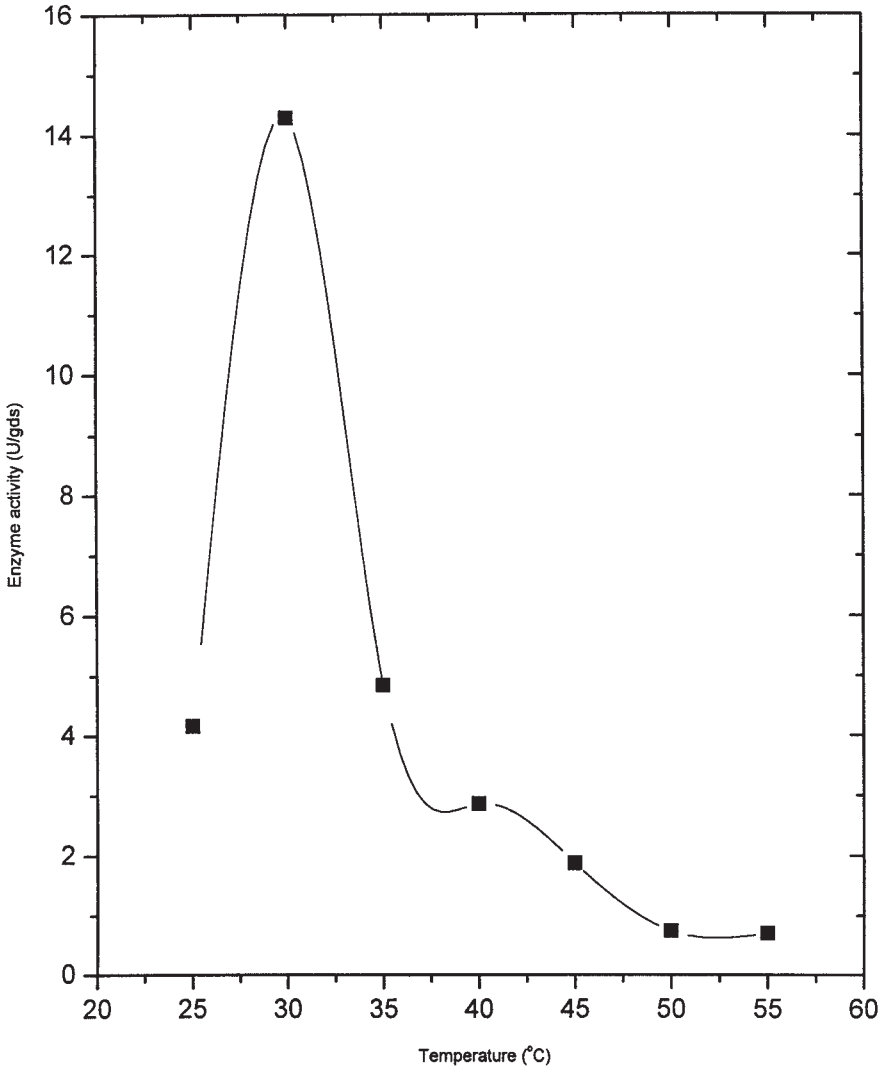

Fig. 2. Effect of incubation temperature on phytase production.

lular enzymes are produced at a growth pH that is near to the optimal pH required for maximal enzyme activity.

Incubation at 30°C was optimum for maximal enzyme production (14.29 U/g of dry substrate) compared with other temperatures (Fig. 2). Temperatures above 30°C did not enhance enzyme production compared with lower temperatures. Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield. These factors are largely characteristic of the organism and are species specific.

The substrate:liquid media ratio, which determined the moisture content of substrates, played a crucial role in the solid-state fermentation process, as observed with wheat bran and other natural substrates *(12)*.

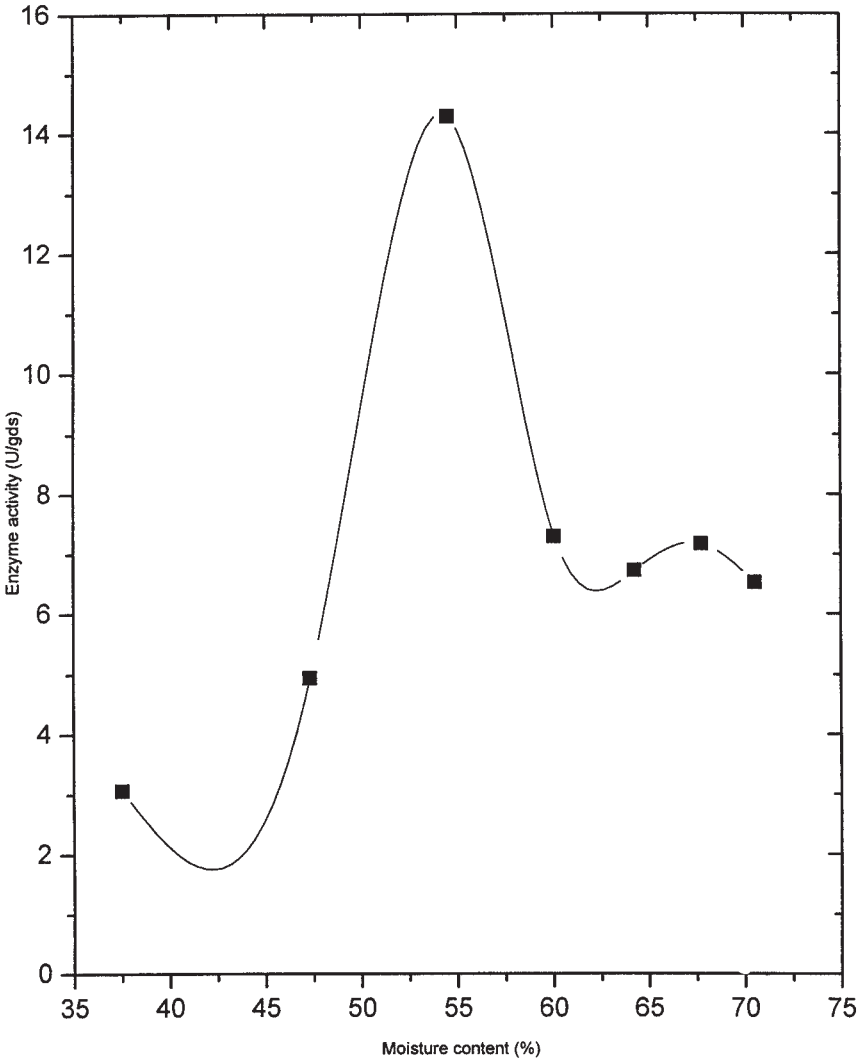

Fig. 3. Effect of initial moisture content of substrate on phytase production.

The optimal level of initial moisture content in the solid support system required for enzyme production during solid-state fermentation was determined by altering the volume of moistening medium added to the substrate, such that different moisture levels were achieved. Figure 3 reveals that the enzyme production increased along with an increase in moisture content from 3.0 U/g of dry substrate at 6 mL (37.5% moisture content) to a maximum of 14.29 U/g of dry substrate at 12 mL (54.5% moisture content). Moisture content levels above 54.5% inhibited enzyme production by *R. oligosporus*, because of accumulation of substrate particles, resulting in inhibition of respiration and consequent reduction in enzyme production.

Spore inoculum concentration influenced the rate of phytase synthesis by *R. oligosporus*. There was a gradual increase in the synthesis of enzyme

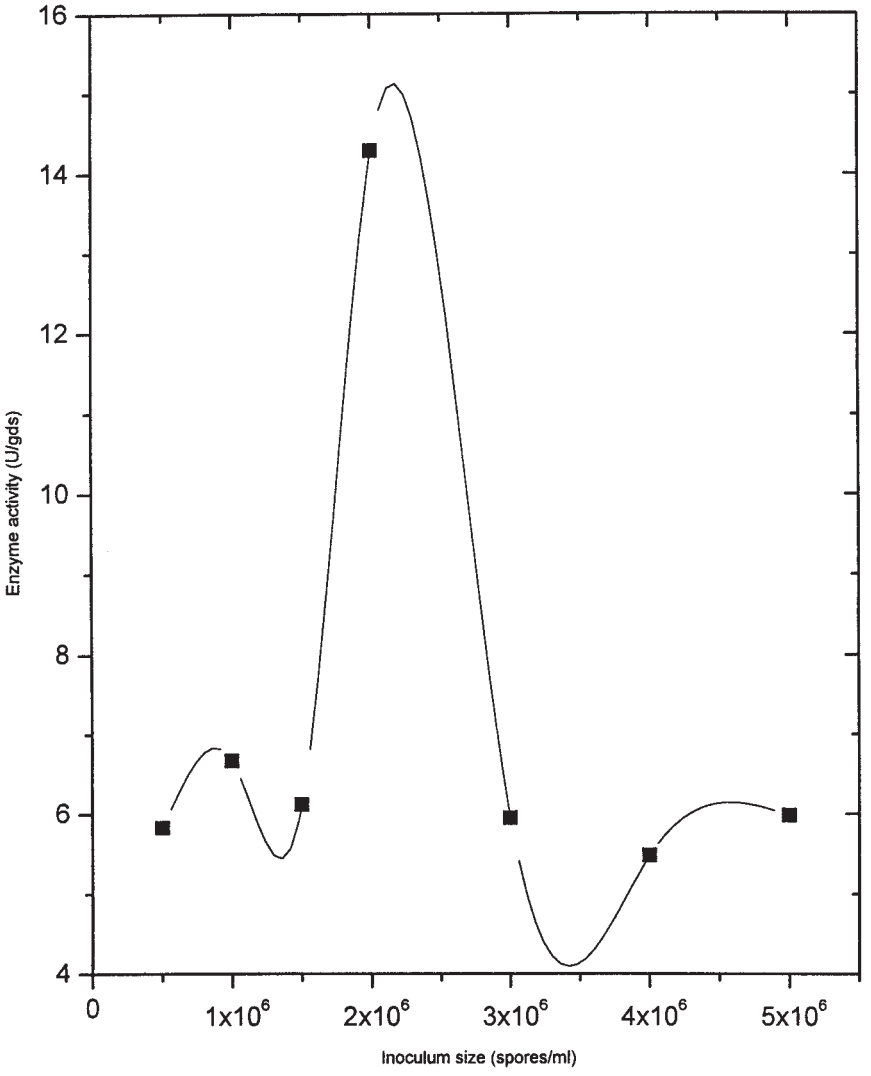

Fig. 4. Effect of inoculum size on phytase production.

along with increase in concentration of spore inoculum up to 2×10^6 spores (14.29 U/g of dry substrate) (Fig. 4). However, spore concentrations above this level resulted in a marginal decline in enzyme production. Nevertheless, considerable levels of enzyme production were observed at other spore inoculum concentrations tested. The results obtained indicated that the concentration of inocula was critical for achieving maximal enzyme production and that it has to be added at optimal level. Perhaps higher concentrations may lead to competition for nutrients in the medium and consequent reduction in enzyme production level. Lower concentrations may not be sufficient to effect maximal enzyme production.

The requirement for additional carbon sources, in the fermentation medium, for enhanced enzyme production was evaluated by incorporat-

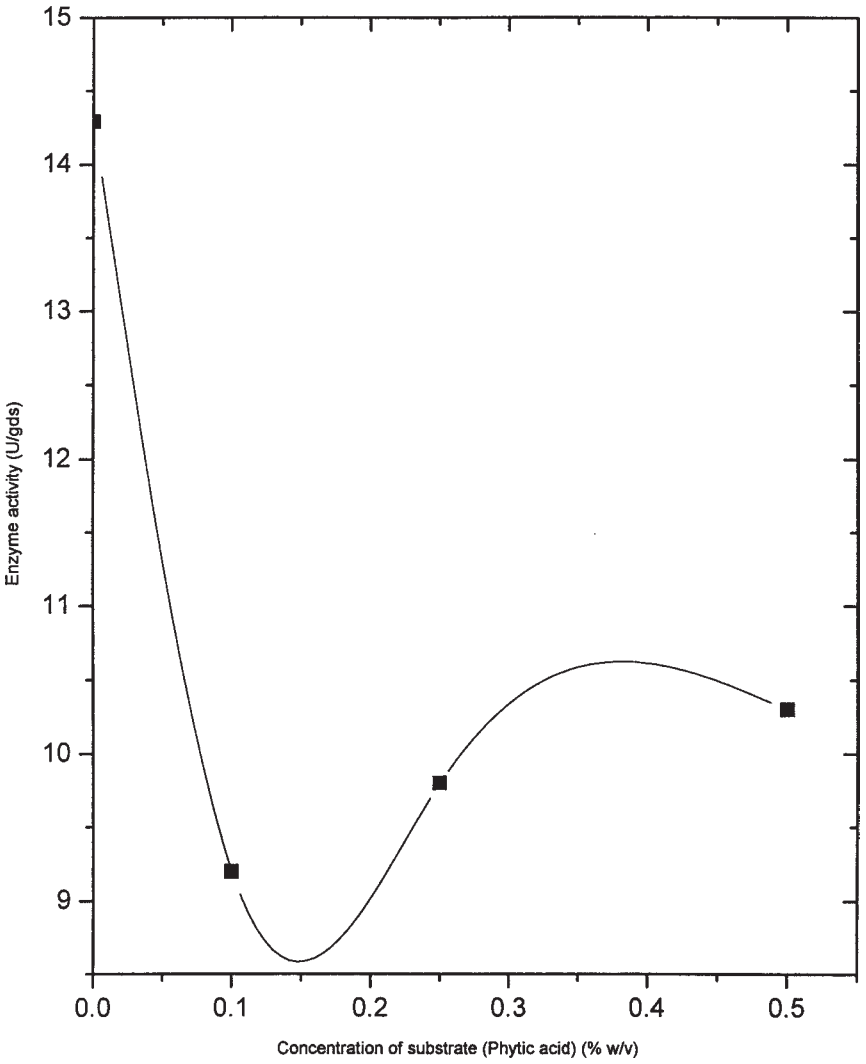

Fig. 5. Effect of addition of phytic acid on phytase production.

ing different carbon sources into the substrate. The results indicated that among the various carbon sources tested, none of them could enhance enzyme yield, and, in fact, many inhibited enzyme production. This clearly shows the direct utilization of the available carbon sources by *R. oligosporus* for its growth, and the results also indicated that the production of phytase is not growth associated. Similarly, the impact of additional nitrogen sources on enzyme production was evaluated by incorporating various organic and inorganic nitrogen sources in medium at the 1% (w/v) level. Results from this experiment also indicated that on addition of nitrogen sources there was a reduction in enzyme yield.

Since phytic acid is the substrate of phytase, different concentrations of the same were tested to evaluate the induction of phytase by *R. oligosporus*

under solid-state fermentation. The results (Fig. 5) indicated considerable reduction in phytase yield on addition of phytic acid. The reason might be the use of coconut oil cake as the substrate, which supplies an adequate amount of phytic acid to the fungal strains. This also indicates the constitutive nature of the enzyme.

Coconut oil cake, one of the major agricultural byproducts of India that is used as a feed material now finds a different use. Solid-state fermentation on coconut oil cake is an economically viable bioprocess for the production of this important feed enzyme, and the fermented matter that is enriched with protein can be used as an excellent feed devoid of phytase.

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