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Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*

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Abstract

Comparisons were made for phytase production using wheat bran (WB) and oilcakes as substrates in solid-state fermentation (SSF) by *Mucor racemosus* NRRL 1994. WB was also used as mixed substrate with oil cakes. Sesame oil cake (SOC) served as the best carbon source for phytase synthesis by the fungal strain as it gave the highest enzyme titres (30.6 U/gds). Groundnut oil cake (GOC) also produced a reasonably good quantity of enzyme (24.3 U/gds). Enzyme production on WB was surprisingly much less (almost 3.5 times less in comparison to SOC). Mixing WB with SOC (1:1 ratio) resulted in better phytase activity (32.2 U/gds). Optimization of various process parameters such as incubation time, initial moisture content and inoculum concentration was carried out using the single variable mode optimization technique. Under optimized conditions, the production of phytase reached 44.5 U/gds, which was almost 1.5-fold higher than the highest yield obtained with any individual substrate used in this study and was more than 4-fold higher than that obtained from WB.

Keywords: Phytase; Solid-state fermentation; *Mucor* sp.; Wheat bran; Oil cakes; Process optimization; Feed enzyme

1. Introduction

Phosphorus (P) is an important element in the feeding of pigs and poultry and is present as organic P, especially in the form of phytic acid and phytate. Phytate (myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate) is the storage form of phosphorus in plants and represents 50–85% of total phosphorus. Phytate P is not absorbed by monogastric animals due to the absence of the enzyme phytase in their intestines. Inorganic or nonphytate P is therefore added to the feed to meet the demands. Unavailable phytate P is excreted in the manure and the excess P from the manure can be washed into waterways, where it causes rapid algal growth (eutrophication) and can affect the amount of oxygen in water, leading to death of fish, hypoxia, etc. (Mallin, 2000). Phytase is an acid phosphohydrolase, which catalyzes the hydrolysis of phosphate from phytic acid to inorganic phosphate and myo-inositol phosphate derivatives. Phytase was discovered by Suzuki et al. in rice bran when they isolated inositol and orthophosphoric acid as the reaction products from phytic acid in 1907 (Nagai and Funahashi, 1962). Two classes of phytase are recognized based on the position of the first phosphate hydrolyzed, namely 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26) (Cosgrove, 1980), of which 3-phytase (myo-inositol-hexakisphosphate-3-phosphohydrolase) is mainly of microbial

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origin whereas 6-phytase is derived from plants. Phytases are classified as the family of histidine acid phosphatases (Piddington et al., 1993), seldom have a similar structure and the cleavage of phosphate groups from the phytic acid is also not by the same mechanism. The use of filamentous fungi for the production of commercially important products through solid-state fermentation (SSF) has gained much research interest during recent years (Pandey, 1992a; Pandey et al., 2000, 2001a; Papagianni et al., 1999). SSF has several advantages over submerged fermentation (SmF), which include lower wastewater output, reduced energy requirements, simpler fermentation media, easier aeration, reduced bacterial contamination etc. (Pandey et al., 2000). SSF has been in particular useful for the production of industrial enzymes (Pandey et al., 1999). Phytase may be produced in SSF by filamentous fungi on selected feed ingredients and the crude product may be mixed in feed rations as a value-added supplement (Pandey et al., 2001a; Bogar et al., 2003). Although several strains of bacteria and yeast can produce phytase, the use of filamentous fungi has been preferred in SSF systems. Fungal phytase has received more attention due to high production yields and acid tolerance for feed production (Kim et al., 1998). Nampoothiri et al. (2004) studied the production of thermostable phytase by a thermophilic strain *Thermoascus aurantiacus* in submerged fermentation and optimized the various process parameters for enzyme production using a semi-synthetic medium supplemented with either wheat bran or wheat bran extract. Various filamentous fungi such as *Mucor*, *Aspergillus*, and *Rhizopus* sp. have been reported to produce phytase and accessory enzymes by SSF. Although several strains of bacteria, yeasts and fungi have been used for production of phytase, two strains of *Aspergillus* sp., *A. niger* and *A. ficuum*, have most commonly been employed for commercial production (Pandey et al., 2001a; Sabu et al., 2005).

The objective of the present study was to determine the effect of different substrate combinations on the production of phytase by the fungal strain *Mucor racemosus*, as well as to optimize the various process parameters that influence the enzyme synthesis. Various process parameters influencing phytase production were evaluated by single parameter optimization studies. The present study aims at the production of an eco-friendly feed enzyme—phytase through SSF.

2. Methods

2.1. Microorganism and inoculum preparation

M. racemosus NRRL 1994 was used for the present study. The culture was grown and maintained on potato-dextrose-agar (PDA) slants. The slants were stored at 4 °C and sub-cultured fortnightly. Five-day-old fully sporulated slant was used for inoculant preparation. For this, 10 ml sterile distilled water containing 0.1% Tween-80 was added to the slant and spores were scraped with a sterile needle. The inoculant obtained contained 5×10^6 spores per ml.

2.2. Substrates

Wheat bran (WB), coconut oil cake (COC), sesame oil cake (SOC), groundnut oil cake (GOC), palm kernel cake (PKC) and olive oil cake (OOC) were used as substrates for the phytase production. WB, SOC and COC were obtained from a local market, Trivandrum. GOC was obtained from Rajkot, Gujarat. PKC and OOC were obtained from Malaysia and Greece, respectively.

2.3. Solid-state fermentation

Five grams of the dried substrate taken in a cotton-plugged 250 ml Erlenmeyer flask were supplemented with 3.0 ml of salt solution containing (%) NH_4NO_3 0.5, $MgSO_4 \cdot 7H_2O$ 0.1 and NaCl 0.1. The substrate moisture was adjusted to the required level by adding distilled water. Substrates were sterilized at 121 °C and 15 psi for 15 min, cooled and inoculated with 1.0 ml spore suspension (5×10^6 spores per ml) of *M. racemosus* strain. The flasks were incubated at 30 °C for 96 h unless otherwise mentioned. All experiments were carried out in two sets.

WB and oil cakes were also used in combination (1:1, w/w) for phytase production. For these experiments, substrate initial moisture was 60.5% and fermentation was carried out for 72 h. The combined substrate that gave the best enzyme yield was selected for further experiments. The results reported are the average of two sets of experiments and standard deviation was ±<5%.

2.4. Optimization of process parameters

SSF was carried out to optimize various process parameters influencing phytase production. These included incubation time (0–144 h), inoculum concentration (0.5–3.0 ml) and initial moisture content of the substrate (52–68%). In further experiments, the medium was supplemented with different carbon sources (lactose, glucose, sucrose, maltose, mannitol and starch at 1.0%—w/w) and different nitrogen sources (ammonium nitrate, ammonium sulphate, sodium nitrate, yeast extract, peptone and corn steep solid at 1.0%—w/w). The effect of varying starch concentration (0.5–3.0% (w/w)) on phytase production was also determined.

2.5. Enzyme extraction

Enzyme extraction was carried out using distilled water with 0.1% Tween-80. Known quantities (wet

wt) of fermented substrates were mixed thoroughly with the required volume of distilled water (so that the final extraction volume was 100 ml) by keeping the flasks on a rotary shaker at 180 rpm for an hour. The suspension was centrifuged at $8000 \times g$ for 20 min and the clear supernatant obtained was assayed for phytase activity.

2.6. Phytase assay

Phytase activity was assayed by measuring the amount of inorganic phosphorus released from sodium phytate solution using the method of Harland and Harland (1980). One unit of enzyme activity was defined as the amount of phytase required to release one micromole of inorganic phosphorus per minute under the assay conditions.

2.7. Protein estimation

Soluble protein content of the crude samples was determined spectrophotometrically according to the method described by Lowry et al. (1951) using bovine serum albumin as standard.

2.8. Biomass estimation

Fungal biomass estimation was carried out by determining the *N*-acetyl glucosamine released by the acid hydrolysis of chitin present in the cell wall of the fungi (Sakurai et al., 1977). For this, 0.5 g (dry wt) of fermented matter was mixed with concentrated sulphuric acid (2 ml) and the reaction mixture was kept for 24 h at room temperature (30 °C). This mixture was diluted with distilled water to make a 1 N solution, autoclaved for 1 h, neutralized with 1 N NaOH and the final volume was made up to 100 ml with distilled water. The solution (1 ml) was mixed with 1 ml acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 ml) was added followed by the addition of 1 ml Ehrlich reagent and the resulting mixture was incubated at 65 °C for 10 min. Once cooled the optical density of the reaction mixture was read at 530 nm against a reagent blank (Blix, 1948). Glucosamine (Sigma) was used as the standard. The results obtained are expressed as mg glucosamine per gram dry substrate (gds).

3. Results and discussion

SSF was carried out for the production of phytase using a *M. racemosus* strain and an effort was made to optimize the important physical, chemical and nutritional parameters that influence phytase production.

3.1. Evaluation of substrates for phytase production

The physical support and the energy required for a fungus to grow and produce the desired metabolite is primarily provided by a substrate (Pandey et al., 2001b). The results obtained are shown in Figs. 1 and 2. Evidently SOC served as the best carbon source for phytase synthesis by *M. racemosus* as it gave the highest enzyme titres (30 U/gds). GOC too produced a reasonably good quantity of enzyme, which was 24.3 U/gds. Enzyme production on WB was surprisingly much less

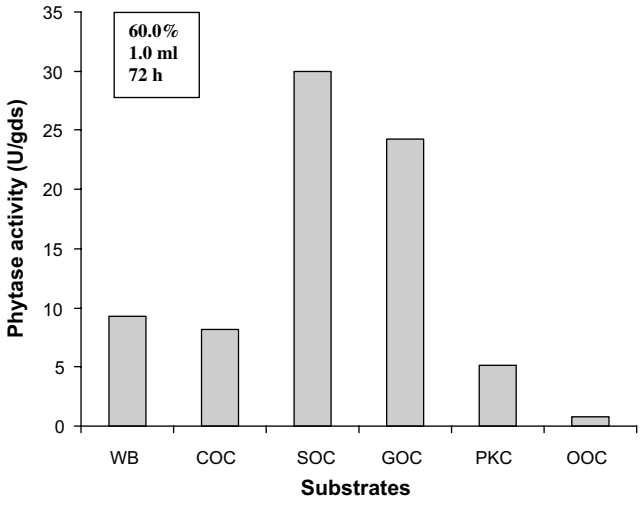

Fig. 1. Phytase production on different substrates. WB—wheat bran, COC—coconut oil cake, SOC—sesame oil cake, GOC—groundnut oil cake, PKC—palm kernel cake, OOC—olive oil cake.

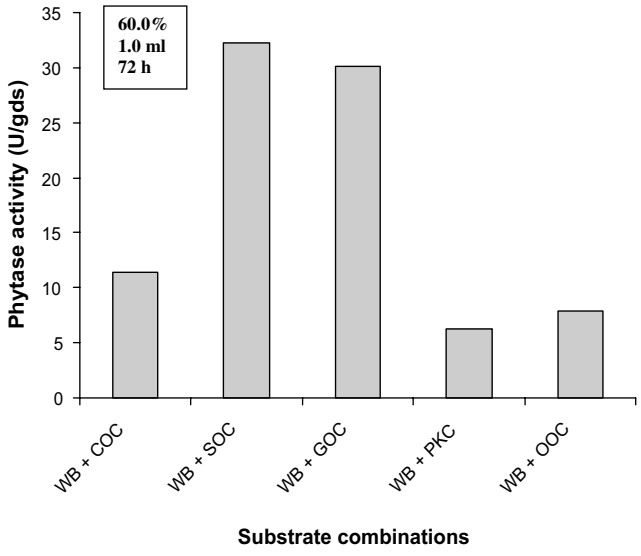

Fig. 2. Phytase production on combination of WB with oil cakes. WB—wheat bran, COC—coconut oil cake, SOC—sesame oil cake, GOC—groundnut oil cake, PKC—palm kernel cake, OOC—olive oil cake.

(almost 3.5 times less in comparison to SOC). This was in contrast to many reports, which have described wheat bran as a potential substrate for phytase production (Pandey et al., 1999, 2000; Kim et al., 1998). However, present results supported the findings of Bogar et al. (2003) who reported the superiority of COC over wheat bran for phytase synthesis.

Although COC and PKC also resulted in enzyme production, the quantities (8.2 and 5.1 U/gds respectively) were much less than with SOC and GOC. Among all the substrates, OOC gave the lowest enzyme activity of 0.83 U/gds, which indicates that it is not at all a good substrate for phytase production. These results could be considered interesting as they indicated the suitability of oil cakes as substrates in comparison to WB, which has generally been considered an ideal substrate for enzyme production in SSF (Pandey et al., 1999).

Fig. 2 shows phytase production by the fungal culture using mixed substrate i.e., WB with oil cakes in a 1:1 (w/w) ratio. Combination of WB with COC, SOC and GOC resulted in an increase in enzyme production. Maximum phytase was produced with WB + SOC (32.2 U/gds), hence mixed substrate was used for further studies.

3.2. Biomass growth and phytase production at different periods of fermentation

Fig. 3 shows the fungal biomass growth and phytase production as well as the soluble protein content at different time intervals. It shows that the enzyme production increased progressively with incubation time and maximal enzyme production was obtained after 96 h (32.8 U/gds). Apparently enzyme production was growth associated and the maximum growth (122.7 mg/gds) was also observed after 96 h. The enzyme yield declined during further incubation, which could have been due to the reduced nutrient level of the medium. Results also showed that the soluble protein content increased along with the enzyme production and a maximum of 102.07 mg/gds soluble protein was obtained after 120 h.

3.3. Effect of inoculum and moisture content on phytase production

There was a gradual increase in the enzyme synthesis with increase in inoculum concentration up to 1.0 ml, but thereafter a steady decline was observed for further inoculum concentrations. From Fig. 4, it can be seen that 1.0 ml of the spore suspension was found best for phytase production, giving the maximum enzyme yield of 32.6 U/gds. Higher concentrations of inoculum were inhibitory for phytase production and minimum enzyme activity (16.09 U/gds) was obtained with the highest inoculum concentration (2.5 ml). Higher concentration of inoculum results in increased competition for carbon source and nutrients (could lead to fungal biomass production), which leads to exhaustion of nutrients and this imbalance results in reduced enzyme production. Ramachandran et al. (2005) reported similar findings with *Rhizopus* sp.

Initial moisture content is a critical factor for growth and enzyme production. Moisture is a factor that is necessary for new cell synthesis and hence is intimately related to SSF (Pandey, 1992a,b). Different initial moisture content of the SSF medium was established by altering the volume of moistening solution added to the solid substrate. Fig. 4 indicates that maximum enzyme production was obtained with 60% moisture content (32.7 U/gds). Reduced enzyme production resulted from further increase in initial moisture content. The

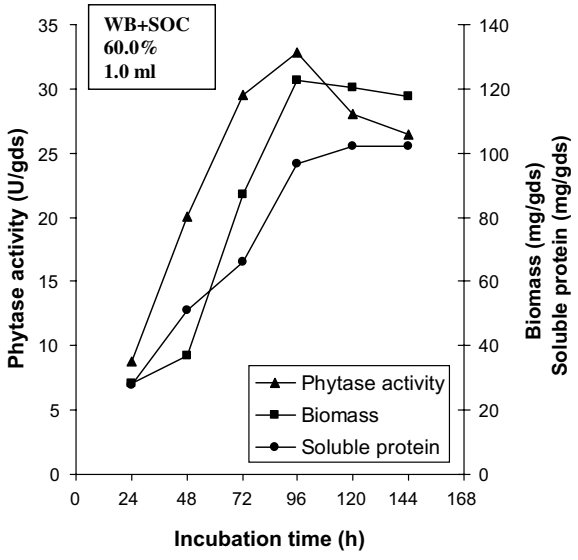

Fig. 3. Phytase activity, growth and soluble protein content at different time intervals.

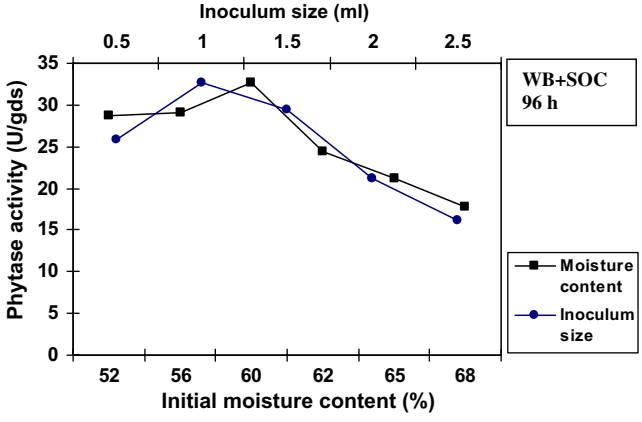

Fig. 4. Effect of inoculum size concentration and initial moisture content on the production of phytase.

air content of the substrate decreases with increasing water content and constant substrate volume (Gautam et al., 2002), which in turn interferes with the microbial activity.

3.4. Effect of supplementation of additional carbon and nitrogen sources on phytase production

Type and nature of carbon and nitrogen sources are among the most important factors for any fermentation process (Pandey et al., 2001b). Carbon source represents the energy source that will be available for the growth of the microorganism. Fig. 5 shows the effect of supplementation of the substrate with mono-, di- and polysaccharides on enzyme production. Evidently, except for maltose, all the compounds exerted a beneficial impact on phytase synthesis by the fungal culture. Other carbon sources gave improved enzyme production and the order of effectiveness was starch > glucose > lactose > sucrose > mannitol. A maximum enzyme activity of 40.3 U/gds was obtained with 1% starch. Vats and Banerjee (2002) also reported that starch was a good substrate for phytase production by *A. niger*, although no or little enzyme production was found with simple sugars. Thus, the findings presented here are in partial agreement with their results. There are reports that fungi such as *Mucor*, *Aspergillus* and *Rhizopus* sp. produce phytase and accessory enzymes by SSF (Pandey et al., 2001a). Hence the accessory enzyme production could have resulted in better utilization of starch, which enhanced the phytase production.

None of the nitrogen sources supplemented was found to enhance phytase production (Fig. 6). However, among the compounds supplemented, one of the inorganic nitrogen sources, sodium nitrate, resulted in 34.09 U/gds enzyme, which was better than the control of mixed substrate without addition of carbon source. However, this was comparatively less if substrate with carbon source was considered as control (40.27 U/gds). Strong repression of enzyme was observed in the presence of nitrogen source, which could have been due to an imbalance in the C/N ratio required for enzyme production. Corn steep solid was found to be highly inhibitory for enzyme synthesis (19.6 U/gds).

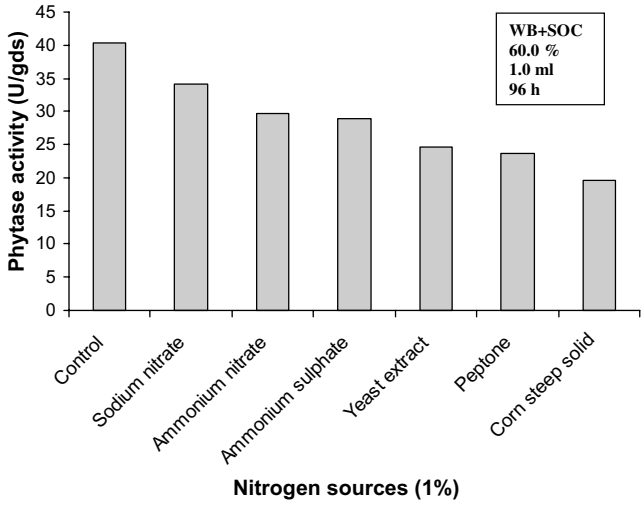

Fig. 6. Effect of supplementation of various nitrogen sources on phytase production.

Since starch was found to enhance the enzyme production, different concentrations of starch were added to the SSF medium to evaluate its enhancement of phytase production. Addition of higher concentration, viz. 2.0%, further improved enzyme yield (44.5 U/gds). However, further increase in starch concentration was inhibitory for enzyme synthesis (Fig. 7).

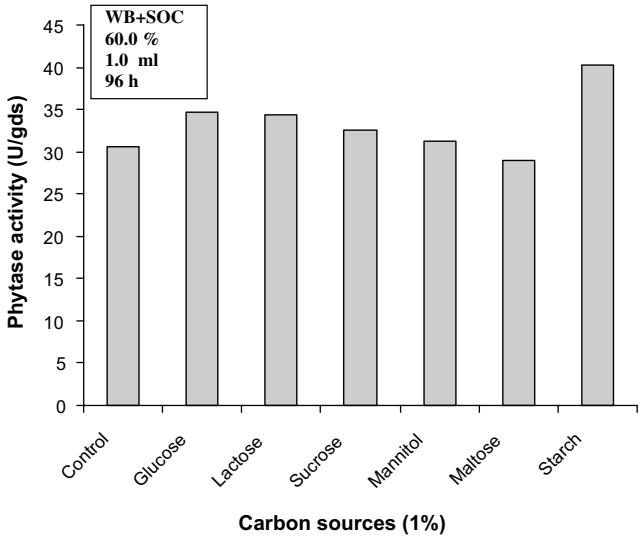

Fig. 5. Effect of supplementation of additional carbon sources.

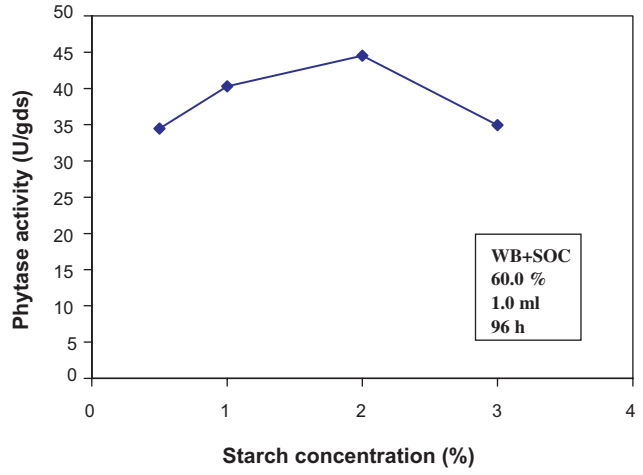

Fig. 7. Effect of varying concentration of starch on phytase production.

4. Conclusions

WB supported cultivation of *M. racemosus* in SSF for phytase production but the yields were not as high as those obtained from oil cakes such as SOC. Combination of WB and SOC proved efficient for enhancing enzyme yield. Addition of starch to the fermentation medium at 2.0% enhanced phytase production.

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