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Microbial production of extra-cellular phytase using polystyrene as inert solid support

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Abstract

Aspergillus ficuum TUB F-1165 and *Rhizopus oligosporus* TUB F-1166 produced extra-cellular phytase during solid-state fermentation (SSF) using polystyrene as inert support. Maximal enzyme production (10.07 U/g dry substrate (U/gds) for *A. ficuum* and 4.52 U/gds for *R. oligosporus*) was observed when SSF was carried out with substrate pH 6.0 and moisture 58.3%, incubation temperature 30 °C, inoculum size of 1.3×10^7 spores/5 g substrate, for 72 h for *A. ficuum* and with substrate pH 7.0 and moisture 58.3%, incubation temperature 30 °C, inoculum size of 1×10^6 spores/5 g substrate for 96 h for *R. oligosporus*. Results indicated scope for production of phytase using polystyrene as inert support.

1. Introduction

Solid-state fermentation (SSF) is generally defined as the growth of microorganisms on solid substrates in the absence or near absence of free water (Pandey, 1992a, Pandey et al., 2000, 2001). SSF has several advantages over submerged fermentation (SmF), which include higher product titers, lower waste water output, reduced energy requirements, simplicity, absence of foam formation, simpler fermentation media, smaller fermentation space requirement, easier aeration, reduced bacterial contamination, high reproducibility, absence of rigorous control of fermentation parameters, less complex plant, use of less water and, since spores are used directly, inoculum tank can be avoided (Pandey et al., 2000).

The use of nutritionally inert materials for SSF facilitates accurate designing of media, monitoring of process parameters, scaling-up strategies and various engineering aspects, which are either impossible or difficult with conventional SSF using organic solid substrates such as wheat bran (Zhu et al., 1994). The inert materials, on impregnation with a suitable medium, provide a homogenous aerobic condition throughout the fermentor, and do not contribute impurities to the fermentation product (Aidoo et al., 1982). It facilitates maximal recovery of the leachate with low viscosity and high specificity for the target product (Prabhu and Chandrasekaran, 1995). Earlier reports indicated that polystyrene, a commercially available insulating and packaging material, could be used as an inert solid support for the production of enzymes (Aidoo et al., 1982; Prabhu and Chandrasekaran, 1995; Sabu et al., 2000). While ion exchange resins (Auria et al., 1990), polyurethane foam (Zhu et al., 1994; Fujishima et al., 1972) and computer cards (Madamwar et al., 1989) have also been used as inert carriers for SSF with fungi, use of polystyrene for phytase production under SSF with fungi has not been reported.

Phytase or myo-inositol hexakisphosphate phosphohydrolase, (EC 3.1.3.8), is an important feed additive to increase the availability of phosphorous and other nutritionally important minerals for monogastric animals by the enzymatic hydrolysis of phytic acid (myo-inositol hexakisphosphate), an anti-nutritional factor present in most of the cereals and legume-based feeds (Pandey et al., 2001; Martinez et al., 1996). The principal end products of phytase action are phosphoric acid and myo-inositol. Most cereals and legumes are rich in protein and fat but the presence of phytic acid discourages their use in food. The phytic acid acts as an anti-nutrient due to its

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chelation of various metals and binding of protein. This diminishes the bioavailability of proteins and nutritionally important minerals.

In the present study, we evaluated the potential of *A. ficuum* TUB F-1165 and *R. oligosporus* TUB F-1166 for phytase production using polystyrene as solid inert support under SSF.

2. Methods

2.1. Microorganisms and inoculum preparation

The fungal strains *A. ficuum* TUB F-1165 and *R. oligosporus* TUB F-1166, obtained from the Culture Collection of the Technical University of Budapest, Hungary, were used in the present study. Both the strains were grown and maintained on PDA slants by cultivating them at 30 °C. Cultures were preserved at 4 °C for short-term storage. To fully sporulated one-week old agar cultures, 10 ml of sterile distilled water with 0.1% Tween-80 was added. Then the spores were scraped using an inoculation needle under strict aseptic conditions. The spore suspension obtained was used as the inoculum. Viable spores in the spore suspension were determined by plate count (colony count) technique.

2.2. Moistening medium

A salt solution having (g/l) ammonium nitrate 5, magnesium sulphate 1, sodium chloride 1, glucose 10, pH-6 was used as the moistening medium.

2.3. Preparation of SSF medium

Five grams of pre-treated (washed thoroughly with distilled water and dried at 60 °C for 24 h) dry polystyrene beads were taken in 250 ml Erlenmeyer flasks, moistened with 7 ml of moistening medium, autoclaved for 20 min at 121 °C (15 lb) and cooled to room temperature before inoculation.

2.4. Inoculation and incubation

Sterilized solid substrate medium was inoculated with 1 ml of the spore suspension (1.3×10^7 spores) for *A. ficuum* and 2 ml (total 2×10^6 spores) for *R. oligosporus* under strict aseptic conditions. The contents were mixed thoroughly and incubated at 30 °C for desired length of period.

2.5. Enzyme extraction

From the fermented solid substrate, enzyme extraction was carried out using 93 ml distilled water with 0.1% Tween-80 so that the final extraction volume was 100 ml (93 ml distilled water + 7 ml moistening medium). First the fermented substrates were properly mixed with distilled water and the flasks were kept on a rotary shaker at 150 rpm for 30 min. After this, the solids were separated from the solution by filtering through a nylon cloth sieve. The solution was centrifuged at 12,000 rpm for 20 min at 4 °C in a refrigerated centrifuge. The supernatant was collected and used for enzyme assay.

2.6. Enzyme assay

Phytase was assayed according to Harland and Harland (1980). One international unit of phytase was defined as the amount of enzyme required for releasing one micromole of inorganic phosphorus at a given temperature and pH. Enzyme yield was expressed as units/gram dry substrate (U/gds)/min.

2.7. Optimization of process parameters for phytase production

The medium described above was taken as a basal medium and the process parameters under study were varied. Incubation time (0–144 h), incubation temperature (25–50 °C), initial pH of the moistening medium (3–9), initial moisture content (37.5–76.1%), inoculum concentration (spore concentration ranging from 2.6×10^6 to 3.9×10^7 spores and 5×10^5 to 4×10^6 spores for *A. ficuum* and *R. oligosporus*, respectively), supplementation with different organic nitrogen sources (beef extract, yeast extract, malt extract and peptone), and inorganic nitrogen sources (ammonium sulphate, ammonium chloride, potassium nitrate and sodium nitrate all at 1% w/v), additional carbon sources (maltose, mannitol, lactose, sucrose, sorbitol and starch at 1% w/v) and phytic acid (0.1–4.0%) were optimized for phytase production. The procedure adopted for optimization of various process parameters influencing phytase production was to evaluate the effect of individual parameters (keeping all other parameters as constant) and to incorporate it at the optimized level in the experiment before optimizing the next parameter. All the experiments were carried out in duplicate and the mean values are reported.

3. Results and discussion

In order to design a suitable bioprocess for maximal phytase production under SSF using a nutritionally inert support, an effort was made to optimize the important physical, chemical and nutritional parameters that influence the production of phytase by the strains of *A. ficuum* and *R. oligosporus* using polystyrene moistened with salt solution as fermentation medium. Results

obtained in the present study showed positive indication of phytase production.

Initially the various process parameters, which influence phytase production under SSF condition, were optimized. The results obtained suggested that phytase production increased progressively along with increase in incubation time until 72 h in the case of *A. ficuum*, where maximal enzyme production (10.07 U/gds) was recorded. In case of *R. oligosporus* phytase production increased until 96 h where maximal enzyme production (2.32 U/gds) was recorded. The enzyme yields declined during further incubation in both the cases. The reason for the decrease in enzyme synthesis after 72 and 96 h could be due to the reduced nutrient level of medium, affecting the enzyme synthesis. Decreased enzyme yield after further incubation could also be due to poisoning and denaturation of the enzyme.

Incubation temperature influenced the rate of phytase production by *A. ficuum* and *R. oligosporus*. Data presented in Fig. 1 indicated that maximal phytase production was observed at 30 °C for both, *A. ficuum* (10.07 U/gds) and *R. oligosporus* (2.84 U/gds). With further increase in temperature, there was a sharp decline in enzyme production.

The pH of medium is the other most important factor for a fermentation process, which influences the microbial growth and activity. Optimum pH required for maximal phytase production during SSF was evaluated using various initial pH levels (3–9) adjusted in the moistening solution. Results presented in Fig. 2 showed that maximal enzyme production was recorded with the pH of the mineral salt solution as 6.0 (10.08 U/gds) for *A. ficuum* and 7.0 (4.52 U/gds) for *R. oligosporus*. Any further increase in the pH of the mineral salt solution reduced the enzyme production. A high pH generally leads to poor growth and results in variations in enzyme yield. These factors are largely characteristic of the organism and are species specific. As is evident from the figure, there appeared more than one peak for increase in the enzyme production, which could be attributed to poor or uneven growth of the culture.

Initial moisture content is a critical factor for 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 (Pandey, 1992a,b). Optimum level of initial moisture content in the solid support system required for enzyme production during SSF was determined by altering the volume of moistening solution added to the solids so that different moisture levels were established. Results in Fig. 3 indicated that maximum enzyme production was recorded with 58.3% moisture content (10.07 U/gds) for *A. ficuum* and the same level for *R. oligosporus* (4.52 U/gds). Any further increase in the initial moisture content reduced

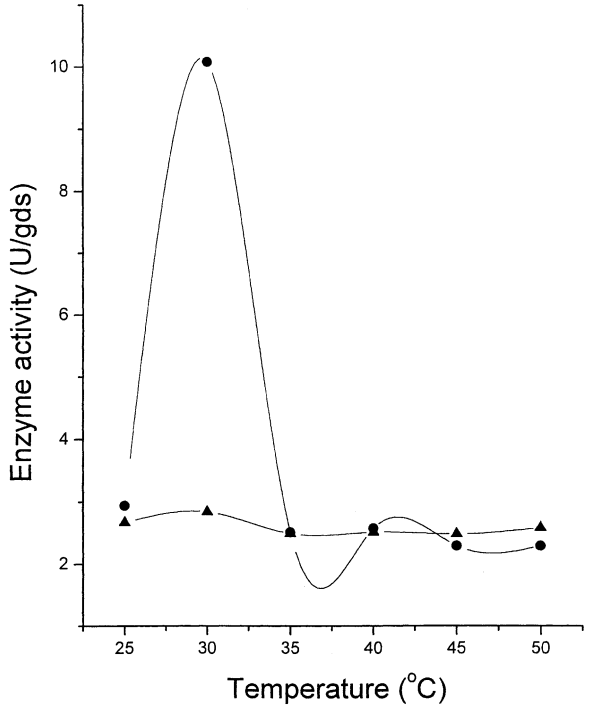

Fig. 1. Effect of incubation temperature on phytase production by *A. ficuum* (-●-) and *R. oligosporus* (-▲-); values shown in all the figures are average of two sets of the experiments.

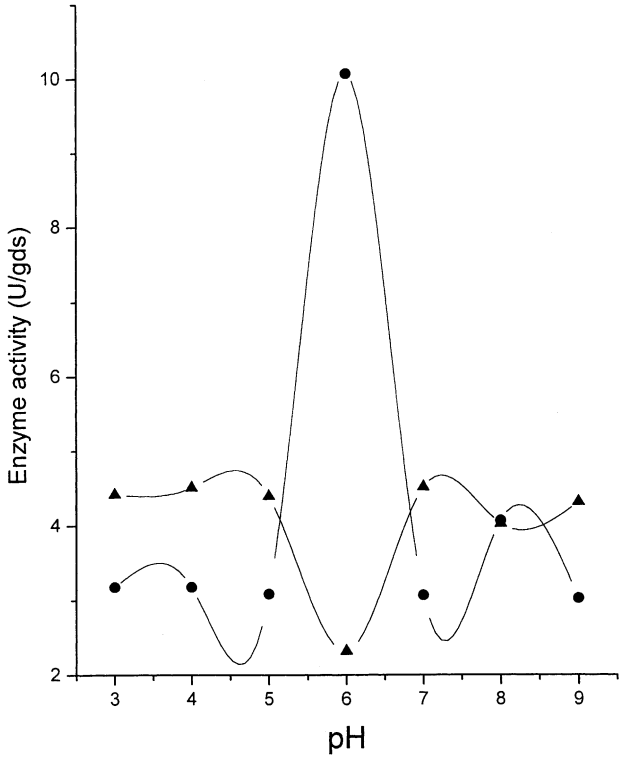

Fig. 2. Effect of initial pH of the substrate on phytase production by *A. ficuum* (-●-) and *R. oligosporus* (-▲-).

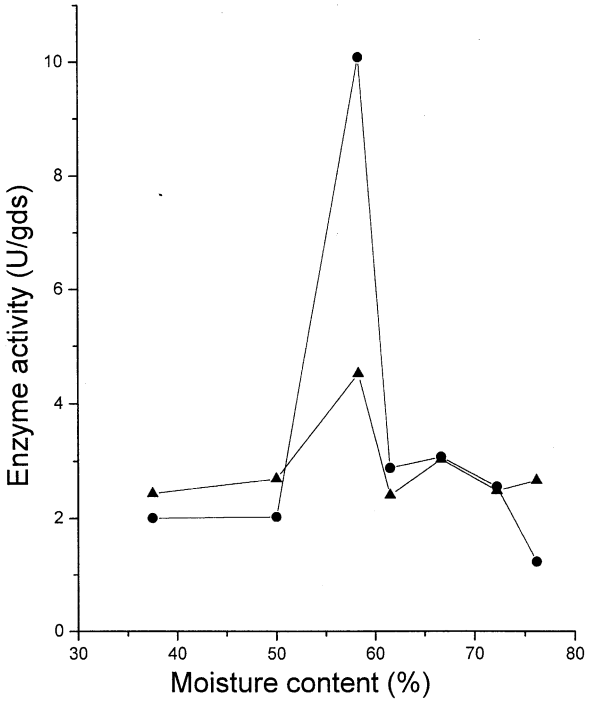

Fig. 3. Effect of initial moisture content of substrate on phytase production by *A. ficuum* (-●-) and *R. oligosporus* (-▲-).

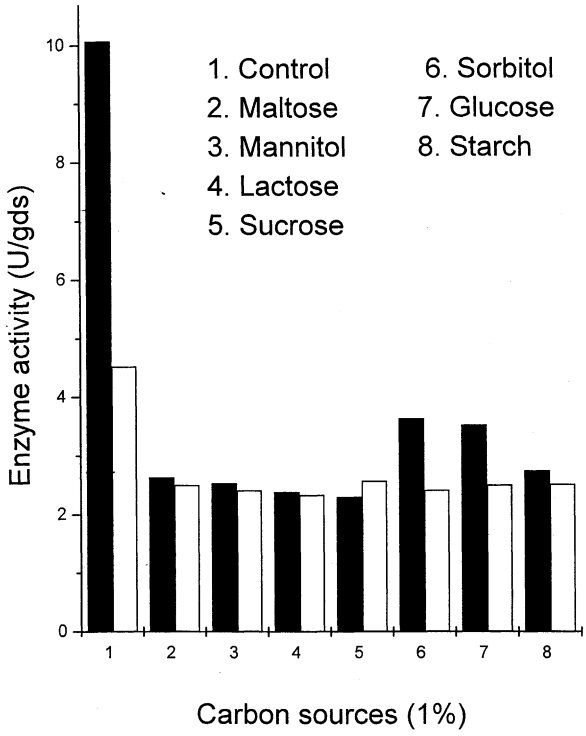

Fig. 4. Effect of supplementation of substrate with additional carbon source on phytase production by *A. ficuum* (■) and *R. oligosporus* (□).

the enzyme production. With increasing water content and constant substrate volume, the air content of the substrate decreases. With both the lowest and the highest water contents, the decomposition rate of the total organic matter was found to decrease. The fungi have shown specific growth optima for various air and water contents of the substrate.

Inoculum concentration also influenced the phytase production. It was observed that there was a gradual increase in the synthesis of enzyme along with increase in concentration of spore inoculum up to 1.3×10^7 spores for *A. ficuum* (10.07 U/gds) (data not shown). However, *R. oligosporus* showed maximal production for 1×10^6 spores (4.52 U/gds).

Impact of supplementation of additional organic and inorganic nitrogen sources to the fermentation medium on enzyme production was evaluated by incorporating various organic nitrogen sources in the medium. It was found that all the four organic as well as inorganic nitrogen sources inhibited the yield of phytase by both *A. ficuum* and *R. oligosporus* (data not shown) and thus there was no need to add external nitrogenous sources in the fermentation medium.

To initiate growth and metabolism in fungi requires carbon sources in easily available form. Hence the effect of addition of additional carbon sources to the fermentation medium on enzyme production was evaluated by incorporating various carbon sources in the medium at 1% w/v level. Fig. 4 indicated that none of the carbon sources were utilized favorably for the production of phytase by either *A. ficuum* or *R. oligosporus.*

Since phytic acid is the substrate of phytase, different concentrations of phytic acid were also added to the fermentation medium to evaluate the induction of phytase by *A. ficuum* and *R. oligosporus* under SSF. Results obtained revealed that there was no increase in yield of enzyme by phytic acid supplementation at the tested concentration (data not shown).

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