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Influence of Inoculum Size on Phytase Production and Growth in Solid-State Fermentation by *Aspergillus niger*

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INFLUENCE OF INOCULUM SIZE ON PHYTASE PRODUCTION AND GROWTH IN SOLID–STATE FERMENTATION BY *ASPERGILLUS NIGER*

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ABSTRACT. *Solid–state fermentation is experiencing renewed interest for industrial enzyme production. Previous studies on the effects of fungal inoculum size on product yield have focused on spore inoculum. However, some organisms require vegetative inocula. This study investigated the effects of initial inoculum colony age, vegetative inoculum size, and duration of fermentation on the production of fungal biomass and phytase in solid–state fermentation using* Aspergillus niger *grown on wheat bran and soy meal. Initial inocula from 7– and 14–day–old potato dextrose agar (PDA) plates were used to study the effect of inoculum colony age in liquid culture and its further influence on fermentation. The liquid inoculum size of (60 – 480 mg of biomass/5 g substrate) and the duration of fermentation were studied for 10 days with periodic sampling every 48 hours. The study was conducted as a replicated full factorial experiment. Statistical analysis showed that phytase production and growth were not affected by inoculum size (over 60 mg/5 g substrate) after 48 hours of fermentation. The duration of fermentation was highly significant with a maximal phytase (1001 ± 94 U/g substrate) and biomass (0.032 ± 0.007 g glucosamine/g substrate) formation at 192 h of SSF. Phytase yield and biomass formation were strongly correlated for the 7– and 14–day plate cultures (the estimated correlation coefficients being 0.87 and 0.73), indicating that phytase production is strongly growth–associated.*

Keywords. *Phytase, Solid–state fermentation,* Aspergillus niger*, Vegetative inoculum size, Plate age, Full factorial design.*

Phytic acid (myo–inositol hexaphosphoric acid) is the main storage form of phosphorus in many legumes and cereals, and up to 80% of the total phosphorus in plants exists as phytic acid (hexaphosphate of myo–inositol) phosphorus (Lolas and Markakis, 1977). It has anti–nutritive properties and forms insoluble complexes with protein and multivalent cations such as Ca^{2+}, Fe^{3+}, and Zn^{2+}, thus reducing their bioavailability (Nair and Duvnjak, 1990). It is also known to inhibit a number of nutritionally important enzymes in vivo (Graf, 1986). Phytic acid can be hydrolyzed chemically, or enzymatically (Ebune et al., 1995) with phytase — a phosphomonoesterase — to inorganic orthophosphate, lower esters of myoinositol, and free inositol (Irving and Cosgrove, 1972), thereby making phosphorus available for bioabsorption. Phytase is present in plants and animal tissues and is produced by many species of fungi and bacteria (Cosgrove, 1966).

Current environmental legislation in Europe restricts the release of phosphorus to the environment, and the U.S. is beginning to enforce similar regulations (Boling et al., 2000). The supplementation of microbial phytase to monogastric animal diets alters the phytic acid complexes; increases the bioavailability of phosphorus, calcium, and protein; and reduces the amount of phosphorus in animal manure (Wodzinski and Ullah, 1996). If phytase were added to the diets of all monogastric animals reared in the U.S., the value of the phosphorus released would be $\$1.68 \times 10^8$ per year, and it would reduce the environmental loading of P by 8.23×10^7 kg (Wodzinski and Ullah, 1996). However, low enzyme yield and the high cost of microbial phytase production have been cited as limiting factors in the use of this enzyme in animal diets (Wodzinski and Ullah, 1996; Orban et al., 1999). Due to the environmental and economic significance of the problem, considerable interest has recently been shown in the production of microbial phytases, which has lead to further investigation of alternative technologies.

Extracellular hydrolytic enzymes and other metabolites are produced in high concentrations in solid–state fermentation (SSF), which has been receiving much attention in the past two decades. SSF involves the growth of microorganisms on moist solid substrates in the absence of free liquid (Cannel and Moo–Young, 1980). SSF is a promising technology for commercial phytase production because it uses unrefined agro–industrial waste products as substrate, involves a less expensive process, can require lower capital investment and operational costs, and results in a higher volumetric productivity over submerged fermentation (SmF). Since the moisture content of the fermented substrate is very low, it can be dried, ground, and used as animal feed, resulting in less waste and less downstream processing (Sato and Sudo, 1999).

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Filamentous fungi are the most important group of microorganisms for solid–state cultivation due to their physiological capabilities and hyphal mode of growth (Mitchell, 1992). Mitchell (1992) has reviewed the importance of inoculum in SSF. However, published work is limited regarding the influence of vegetative inoculum quality for solid–state fermentation, and most of the studies used spore suspensions (Papagianni et al., 1999). Spore inocula are advantageous because they allow greater flexibility in the coordination of inoculum preparation with the cultivation process. They retain viability for longer periods than fungal mycelia, and therefore can be stored and used when required. Spores are also less susceptible than mycelia to any mishandling that might occur between harvesting and inoculation. The disadvantage of spores is that they are metabolically dormant, so the metabolic activities must be induced and the appropriate enzyme systems must be synthesized before the fungus begin to utilize the substrate and grow (Mitchell, 1992). The development of spore inoculum for large–scale fermentations involves an increased number of sub–culturings, which in some cases leads to degeneration. This can be overcome by simultaneous inoculum development in two fermenters, which contributes substantially to the cost of production (Lonsane et al., 1992).

One of the major limitations of SSF is the unique physical conditions for growth, which may be partially overcome by selecting an inoculum size large enough for the mycelial fragments or spores to initially colonize most of the substrate particles without overcrowding and competing for the limited nutrient supply (Abdullah et al., 1985). The inoculum is generally used at a high ratio in most fermentation processes for the production of the desired product in shorter period (Lonsane et al., 1992). Influence of inoculum size on phytase production in SSF using different strains of *Aspergillus* has previously been reported (Nair and Duvnjak, 1990; Al–Asheh and Duvnjak, 1995). Both studies reported that the rate of change of phytic acid conversion of the canola meal depends on the mycelial inoculum size, with a complete reduction of phytic acid after 2 days when inoculated with larger amount of mycelial inoculum. However, they did not specifically quantify phytase production.

Strains of *Aspergillus* are reported to produce the most active extracellular phytases (Shieh and Ware, 1968; Howson and Davis, 1983). The feed industry has been most interested in phytase production by *Aspergillus* sp. (Scott et al., 1999). The *Aspergillus niger* strain selected for this study needs a vegetative inoculum for SSF, because spore production is minimal on the plate culture. Therefore, a vegetative mycelial inoculum was developed in a liquid medium and used as the inoculum for SSF (Krishna and Nokes, 2000, 2001).

In the present study, we investigated: (1) the relationship between vegetative inoculum size (biomass concentration) of *Aspergillus niger* on phytase yield and growth over time in solid–state fermentation, (2) the effect of colony age of the initial inoculum on the maximal phytase yield and growth over time, and (3) the interaction between vegetative inoculum concentration and initial inoculum colony age on phytase yield and growth over time in SSF.

Materials and Methods

Microorganism

A phytase–producing strain of *Aspergillus niger* (provided by Alltech, Inc., Nicholasville, Kentucky) was used throughout this study. Culture maintenance included a bimonthly sub–culture from a molasses agar plate to a potato dextrose agar (PDA) plate, and storage at room temperature (~25°C). Inoculation was accomplished by cutting an agar block of 5×5 mm^2 from the growing edge of the culture and transferring it to the center of the new plate. The PDA plates were incubated for 7 and 14 days, respectively, and used as the inoculum for the liquid culture.

Liquid Inoculum Preparation for SSF

The liquid inoculum for solid–state fermentation was prepared using a complex medium (M1+) comprising: corn starch (28 g/l), glucose (5 g/l), peptone (18 g/l), KCl (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (1.5 g/l), KH_2PO_4 (1 g/l), $CaCl_2 \cdot 2H_2O$ (2 g/l), and wheat bran (20 g/l). One hundred ml of the medium was dispensed into 250 ml Erlenmeyer flasks and sterilized at 121°C for 20 min. The wheat bran was sterilized separately and added to the sterile medium aseptically prior to inoculation. The pH of the medium after sterilization was between 5.0 and 5.3. Addition of wheat bran to the medium increased the initial pH to 5.5 – 5.8. The pH was not adjusted during this study.

The liquid medium flasks were inoculated with 7– or 14–day–old fungal cultures grown on the PDA plate by aseptically transferring a block of mycelium and spores (5×5 mm^2 area) of the plate culture into the flasks. The flasks were incubated in an environmental shaker (New Brunswick Scientific, Edison, New Jersey) at 200 rpm and 30°C for 72 h.

Solid–State Fermentation (SSF)

Solid–state fermentation was carried out in 250 ml flasks containing air–dried substrates (3.5 g of wheat bran and 1.5 g of full–fat soybean flour). The flasks with substrate were sterilized at 121°C for 20 min prior to inoculation. Moisture content was then adjusted to 53% (wet basis) by aseptically adding the appropriate amount of liquid inoculum and then sterile water. The effect of inoculum size on SSF was studied by inoculating different liquid inoculum levels containing biomass (by dry weight) ranging from 60 to 480 mg/5g of substrate. The moisture content was adjusted accordingly to obtain a final moisture content of 53% (wet basis). The flasks were incubated at 30°C for up to 240 h.

Two flasks from each treatment were harvested every 48 h, and 0.5 g of the fermented sample was used for glucosamine analysis. The rest of the sample was used for enzyme extraction. The crude enzyme was extracted by adding 20 ml distilled water and 0.1% (v/v) Tween 80 per gram of initial solid media, homogenizing, and then shaking the suspension at 200 rpm for 1 h at 30°C. The homogenized suspension was filtered through filter paper (No. 1, Whatman Int. Ltd., Maidstone, U.K.), and the clear filtrate was used for the phytase assay. Each experiment was conducted in triplicate with two sub–samples of each treatment. The results of the sub–samples were averaged and used for the statistical analysis.

Analytical Methods

Phytase assay — Phytase activity was determined from the enzymatic hydrolysis of sodium phytate under controlled conditions and measurement of the amount of ortho–phosphate released (Harland and Harland, 1980). One unit of phytase activity (U) was defined as the amount of enzyme required to liberate 1 µmol of inorganic phosphate per minute under standard assay conditions.

Biomass in the liquid culture — Biomass in the liquid culture was measured by dry weight measurements using the method reported by Cui et al. (1998). An aqueous salt solution of Na_2SO_4 (150 g/l) was used to separate biomass and wheat bran. The cells were separated through a pre–dried and pre–weighed glass microfiber filter, (Grade GF/A, Whatman Int. Ltd., Maidstone, U.K.). The separated fungal biomass was oven–dried at 105°C to a constant weight (usually 24 h), and the dry weight was measured and expressed as mg of biomass.

Biomass in the solid culture — Glucosamine, an essential and stable component in the chitin of mycelial cell walls, was used as an indirect method for biomass determination and an efficient parameter for growth in SSF. Glucosamine from the biomass was released by hydrolysis, according to the method of Sakurai et al. (1977), and expressed in gram of glucosamine per gram of fermented substrate.

Experimental Design and Statistical Analysis

A 5× 5 full factorial design of experiments was used at two levels of plate age. The experimental factor levels used were: 60, 100, 180, 320, and 480 mg of inoculum (as biomass by dry weight measurements) per 5 g of substrate, and the duration of SSF ranged from 48 to 240 h with sampling every 48 h. This set was replicated three times with two sub–samples in each set of experiments at two levels of plate ages (7 and 14 days old).

PROC GLM in SAS Software (SAS Institute Inc., Cary, North Carolina) was used to evaluate the main effects and treatment combinations (plate age, liquid inoculum size, and duration of SSF). The dependent variables quantified in this study were phytase production under SSF (units/g substrate), and glucosamine content under SSF (g/g substrate). PROC REG (SAS Software) was used to fit the model by least squares and to predict the relationship between phytase activity and glucosamine content in solid–state fermentation. All data presented are the mean values and their standard deviation calculated from triplicates with two sub–samples for each treatment.

Results and Discussion

The main effects of individual treatments were investigated by averaging phytase yield over the other factors. The main effect of inoculum size (biomass concentration by dry weight measurements) on phytase production was studied by averaging phytase yield over both colony ages (fig. 1). The main effect of inoculum size on phytase production was significant ($Pr > F = 0.0001$) at 0 and 24 h of SSF, where the phytase production is linearly related to the amount of inoculum added. The main effect of inoculum size on phytase production was also significant ($Pr > F = 0.008$) at 48 h of SSF, where the higher inoculum levels of 320 and 480 mg of biomass yielded 1.56–fold more phytase than the lower inoculum levels of 60, 100, and 180 mg biomass. No significant main effect of inoculum size on phytase was detected during further incubation periods (96 – 240 h) of SSF. In addition, under the imposed environmental conditions, high cell densities did not increase the rate of phytase production; an inoculum level of 60 mg/5g of substrate produced a phytase yield of 971 ±94 U/g substrate within the same time frame (192 h) as an inoculum level of 480 mg/5g of substrate (phytase yield of 1080 ±94 U/g substrate). For fermentation times higher than 48 h, it is sufficient to use less inoculum.

The effect of inoculum colony age (plate culture age) on phytase production was determined by averaging enzyme yield over inoculum size (fig. 2). The main effect of plate culture age on phytase production was not significant at 0 and 24 h of SSF. At 48 h of SSF, the main effect of plate culture age was significant ($Pr > F = 0.019$). The 14–day plate culture averaged over all inoculum size treatment levels yielded 1.32–fold more phytase than the 7–day plate culture. No significant main effect of plate culture age on phytase yield was detected at 96 h of SSF. The main effect of plate culture age was significant ($Pr > F = 0.005$) at 144 h of SSF, where the 7–day plate culture averaged over all inoculum sizes yielded a 1.10–fold increase in phytase over the 14–day plate culture. No significant main effect of plate culture age on phytase yield was detected at 192 – 240 h of SSF, which is the duration that resulted in maximal phytase production. Therefore, the primary inoculum age did not influence phytase yield at maximal production periods, and the younger plate culture would be preferred to speed up cycle time in production.

The preferred fermentation scheme was determined by statistically comparing the treatments (inoculum size and inoculum colony age) at each SSF sampling time. At 48 h of SSF, the treatment combinations were statistically significant ($Pr > F = 0.02$). The treatment combinations that give the maximal phytase production at 48 h of SSF were the 14–day plate culture and the inoculum levels of 100, 180, 320, or 480 mg biomass, which statistically equaled ($\alpha = 0.05$) the 7–day plate culture at higher inoculum levels of 320 or 480 mg with an average phytase production of 193.83 U/g

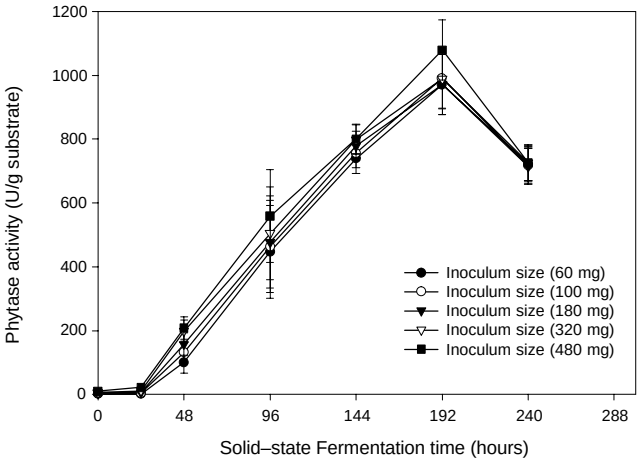

Figure 1. Main effect of inoculum size on phytase production of solid–state fermentation experiment (phytase yield averaged over both plate cultures) using *Aspergillus niger* on wheat bran and soy meal substrate. Data sorted by inoculum size treatments.

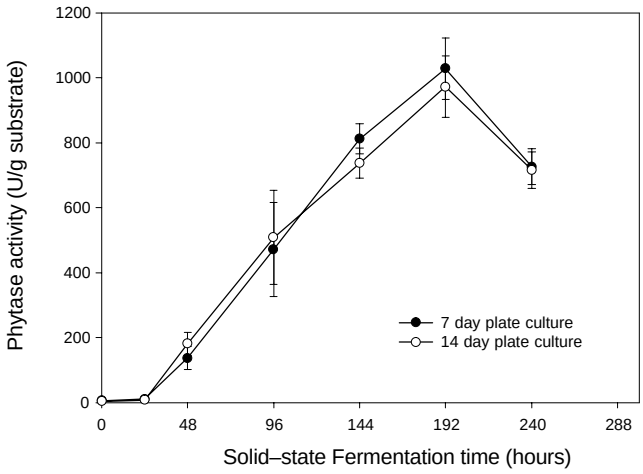

Figure 2. Main effect of plate culture age on phytase production of solid–state fermentation experiment (phytase yield averaged over all the inoculum size treatments) using *Aspergillus niger* on wheat bran and soy meal substrate. Data sorted by plate culture age.

substrate. At 96, 144, 192, and 240 h of SSF, the treatments were not statistically different ($\alpha = 0.05$) from each other for phytase production. The treatment combinations which gave the maximal phytase production value was the 7–day plate culture (1028.8 ±94 U/g substrate), which statistically equaled ($\alpha = 0.05$) the 14–day plate culture (972.5 ±94 U/g substrate), both with all levels of inoculum treatments at 192 h of SSF. Thus, the maximal phytase production value in this study calculated as the mean phytase production of the treatment combinations was 1001 ±94 U/g substrate.

The main effect of inoculum size on glucosamine production was studied by averaging glucosamine content over both plate culture ages. The main effect of inoculum size on glucosamine production was significant ($\alpha = 0.05$) at 0 to 48 h of SSF. Higher inoculum levels of 320 and 480 mg yielded more glucosamine than the lower inoculum levels. No significant main effect of inoculum size was observed at further incubation periods (96 – 240 h) of SSF.

The main effect of colony age on glucosamine content was determined by averaging glucosamine production over inoculum size treatments (data not shown). No significant main effect of plate culture age on glucosamine content was observed at any duration of SSF.

The interaction between the treatments (inoculum size and plate culture age) for growth was determined by statistically comparing the glucosamine production with each treatment level at each SSF sampling time. The treatments were not statistically different ($\alpha = 0.05$) from each other at any duration of SSF. The two–way interaction between initial inoculum colony age and inoculum size was not significant for phytase or glucosamine production at any duration of SSF.

The main effect of SSF time on phytase yield was observed by averaging phytase yield over both plate culture ages and inoculum size. Duration of fermentation was highly significant ($Pr > F = 0.0001$) for phytase production. Phytase production was significantly higher ($\alpha = 0.05$) at 192 h of SSF with a mean phytase yield of 1001 ±78 U/g substrate. An ANOVA of the overall experiment is presented in table 1.

The main effect of SSF time on biomass yield (glucosamine content) was observed by averaging glucosamine content over both colony ages and inoculum size. Duration of fermentation was highly significant ($Pr > F = 0.0001$) for biomass production. The highest biomass production occurred at 192 h of SSF (0.032 ±0.007 g/g substrate), which was statistically equivalent ($\alpha = 0.05$) to biomass levels at 144 h of SSF (0.030 ±0.009 g/g substrate).

The rate of phytase and biomass formation was evaluated during the exponential phase of the fermentation process and is presented in table 2. The results show that rate of phytase production in this fermentation process is similar regardless of the amount of inoculum used. Similar results were also observed with the biomass formation rates. The rate of phytase and biomass formation is similar at all inoculum levels, which clearly shows that phytase production or growth is independent of the amount of inoculum used (between 60 mg/5g and 480 mg/5g of substrate).

Since phytase and glucosamine production appeared to be related, a correlation was performed to determine if phytase production could be predicted by glucosamine content. The results of the representative treatment relating phytase and glucosamine are presented in figure 3. A strong and highly significant correlation ($R^2 = 0.87$ and $Pr > F = 0.0001$) between phytase and glucosamine content was observed when the 7–day plate culture was used as the primary inoculum. A fairly strong and highly significant correlation ($R^2 = 0.73$ and $Pr > F = 0.0001$) between phytase and glucosamine content was also observed with the 14–day plate culture.

Figure 4 plots the average phytase production and biomass formation at the different inoculum treatment levels against time of fermentation. This clearly shows that the phytase production only occurs during the exponential growth phase and declines during the stationary phase. This indicates that phytase production is strongly growth–associated. This confirmed our previous observation regarding the relationship between phytase and growth using this strain of *Aspergillus niger* (Krishna and Nokes, 2001). Growth–related phytase production has also been reported by a few

Table 1. ANOVA for the full factorial experiment evaluating the effects of inoculum age (age of plate culture in days), inoculum size (mg dry weight of biomass), and duration of solid–state fermentation (h) on phytase production (U/g substrate) by *Aspergillus niger* on wheat bran and soy meal as substrate.

Source of Variation	df	Mean Square	F value	Pr > F
Age of plate culture	1	3413	0.56	0.4556
Inoculum size	4	14560	2.41	0.0993
Solid–state fermentation time	5	2912670	481.32	0.0001
Age of plate culture × Inoculum size	4	1081	0.18	0.9486
Age of plate culture × Solid–state fermentation time	5	11482	1.90	0.1081
Inoculum size × Solid–state fermentation time	20	2474	0.41	0.9854
Age of plate culture × Inoculum size × Solid–state fermentation time	20	2069	0.34	0.9951
Error	60	6051		

Table 2. Effect of different inoculum size treatments (data averaged over both plate ages) on phytase and biomass production kinetics during the solid–state fermentation process by *Aspergillus niger* on wheat bran and soy meal as substrate.

Inoculum Size Treatents (mg of inoculum/ 5 g substrate)	Phytase Production Rate (U/g substrate/h)	Biomass Production Rate (g glucosamine/g substrate/h)	Maximum Phytase Yield (U/g substrate) (MSE = 94 U/g substrate)	Time Required for the Maximal Phytase Activity in SSF (h)
60	6.15 ±0.37	0.00019 ±0.00005	971	192
100	6.27 ±0.31	0.00023 ±0.00007	992	192
180	6.43 ±0.29	0.00017 ±0.00006	972	192
320	6.56 ±0.21	0.00018 ±0.00005	989	192
480	6.49 ±0.31	0.00021 ±0.00005	1080	192

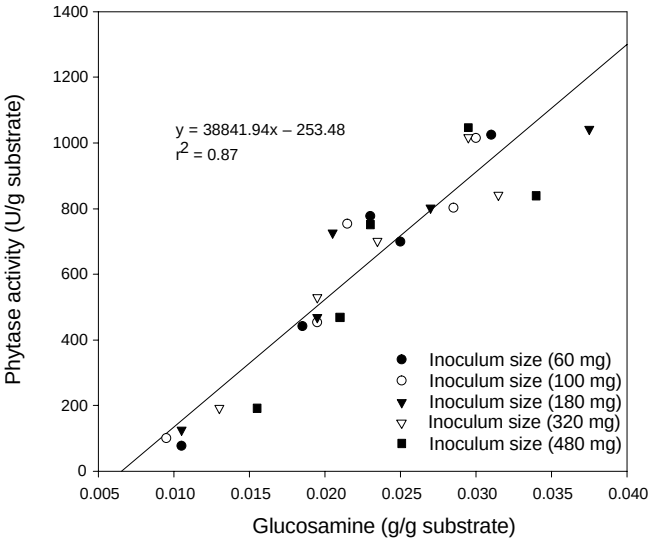

Figure 3. Correlation between phytase production (U/g substrate) and glucosamine content (g/g substrate) in solid–state fermentation inoculated with 7–day–old plate culture. Data sorted by inoculum size treatments.

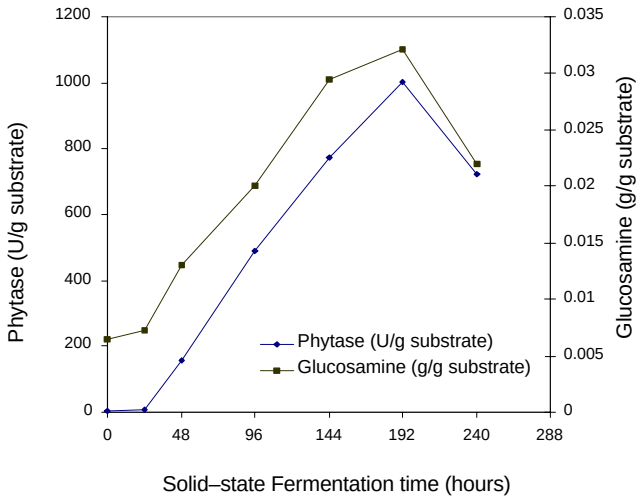

Figure 4. Mean phytase production and growth (biomass formation measured as glucosamine content) of solid–state fermentation experiment using *Aspergillus niger* on wheat bran and soy meal substrate. Data averaged over both plate cultures and different inoculum size treatments.

researchers using a vegetative inoculum from *Aspergillus ficuum* (Ebune et al., 1995) and a spore culture of *Aspergillus niger* (Mandviwala and Khire, 2000).

With this strain of *Aspergillus niger*, spore production on the PDA plate was minimal. Therefore, a vegetative inoculum was developed in a liquid medium by transferring a portion of spores and mycelia from the PDA plate cultures. In the complex liquid medium (with added wheat bran), this strain grew as fine pellets, free mycelial filaments, and clumps and contributed to high phytase yields (Papagianni et al., 1999). In our previous study, we observed that a 72–h–old liquid culture inoculum of this strain of *Aspergillus niger* resulted in maximal phytase production in SSF (Krishna and Nokes, 2001) under similar conditions. Therefore, an unwashed liquid culture along with the liquid enzyme (appropriate amount of liquid inoculum to give the related biomass) from 72–h–old liquid culture grown in a complex medium was used as the inoculum for SSF in the present study.

With the unwashed liquid mycelial inoculum, the fungal hyphae were already induced for enzyme production, and along with the mycelial biomass, the inoculum medium may have contained growth factors and extracellular enzymes that were transferred to the SSF medium. Therefore, the amount of the enzyme transferred to the solid–substrate medium was different with each inoculum size treatment. The smallest amount of inoculum level contains about 0.73 units of phytase for the 7–day plate culture and 0.43 units for the 14–day plate culture, respectively. The largest amount of enzyme that was transferred with the biomass contained proportionally large amounts of enzyme (14.6 units of phytase for the 7–day plate culture and 8.64 units for the 14–day plate culture).

Nair and Duvnjak (1990) reported on the effect of inoculum concentrations on phytase production by using different spore concentrations of *Aspergillus niger* and *Rhizopus oligosporus* inoculated on canola meal in an SSF process. Both organisms showed a higher rate of phytic acid hydrolysis with higher spore concentration. The effect of vegetative inoculum concentrations of *Aspergillus ficuum* on the reduction of phytic acid content of canola meal in an SSF process was also reported by Nair and Duvnjak (1990). They reported that a larger amount of biomass in the inoculum increased the rate of phytic acid hydrolysis. In our study, phytic acid reduction was not measured. However, Ebune et al. (1995) reported that the rate of phytic acid reduction in canola meal in an SSF process is related to the production of phytase. Therefore, in this study we assumed that the phytase production is proportional to phytic acid hydrolysis. A comparison was done with the previous studies, and our experimental results are in contrast to the previous reports related to phytase production and the effect of inoculum concentrations.

While Mandviwala and Khire (2000) reported maximal activity of 108.5 U/g dry moldy bran with cowpea meal as substrate using *Aspergillus niger* in SSF on day 7 of fermentation, our values were 1001 ±94 U/g initial air–dried substrate on day 8, and 775 ±46 U/g initial air–dried substrate on day 6 of SSF. In both the studies, unit activity (U) was

measured as the amount of enzyme that liberated 1 μmol of phosphorus per minute under standard assay conditions. However, a direct comparison could not be made since the expression of units was different, i.e., U/g dry moldy bran and U/g initial air–dried substrate.

CONCLUSIONS

This investigation was focused on the effect of initial inoculum age (plate culture age) and inoculum size, and their interactive effects over the duration of SSF for the production of phytase and biomass using a vegetative inoculum of *Aspergillus niger.* The results from this study indicated that the production of phytase and microbial biomass was not influenced by the inoculum size during SSF after 48 hours of fermentation.

The main effect of duration of SSF averaged over all the treatments was significant for phytase yield and growth, with a maximal phytase production at 192 h of SSF and an average phytase yield of 1001 ±94 U/g substrate at a biomass level of 0.032 ±0.007 g/g substrate. The results indicate that above 60 mg biomass/5 g substrate, the vegetative inoculum size of this specific strain of *Aspergillus niger* is not important for growth or phytase yield in SSF. A good correlation between phytase yield and growth was observed with the different ages of inocula.

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