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THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE AND SUBSTRATE INITIAL MOISTURE CONTENT ON *ASPERGILLUS NIGER* GROWTH AND PHYTASE PRODUCTION IN SOLID–STATE CULTIVATION

S. B. Carter, S. E. Nokes, C. L. Crofcheck

ABSTRACT. *Aspergillus niger is being used commercially for phytase production utilizing solid–state cultivation; however, no studies have been published that investigated the optimal environmental temperature and initial substrate water content to maximize fungal growth and/or phytase production. Solid–state cultivations of Aspergillus niger on wheat bran and soybean meal were conducted at three temperatures (25°C, 30°C, and 35°C) and three initial moisture contents (50%, 55%, and 60% wet basis) in a split–plot full–factorial experimental design. Fermentations were conducted for 0, 24, 48, 72, and 120 h. The containers were sampled destructively and assayed for phytase activity and glucosamine concentration as an estimate of fungal biomass. Temperature affected phytase activity production, but substrate initial moisture content did not. The highest phytase activity was found at 30°C, 50% to 60% initial moisture content, and 72 h of fermentation. Initial substrate moisture content affected glucosamine production after 72 and 120 h of fermentation. The maximum glucosamine was produced at 35°C, either 50% or 60% initial moisture content, and 120 h of fermentation. The results show that the optimal biomass growth conditions are not the same as the optimal phytase production conditions, suggesting that phytase production is not entirely correlated with fungal growth.*

Keywords. Aspergillus niger, *Environmental temperature, Phytase, Solid–state cultivation, Substrate moisture content.*

P hosphorus is an essential mineral for animal growth and development (Poulsen, 2000), stored mainly as phytic acid in cereals (Common, 1940), seeds (Boland et al., 1975), and legumes (Nelson et al., 1968). Phytic acid is not readily assimilated by monogastric animals (Boland et al., 1975; Harland and Harland, 1980) and has antinutritive properties because it forms complexes with proteins and multivalent cations (such as Zn^{2+}, Ca^{2+}, and Fe^{3+}), which reduces their bioavailability (Nair and Duvnjak, 1991). Phytic acid also has been shown to inhibit nutritionally important enzymes *in vivo* (Graf, 1986). The nutritional impediments caused by phytic acid result in higher levels of phytate phosphorus being released into the environment via animal excrement. Phosphorus in the environment is associated with eutrophication of fresh waters and is a major problem in surface water quality (Commission of the European Communities, 1992; USEPA, 1996).

Phytic acid can be hydrolyzed chemically, which often degrades the nutritional value of the feed, or enzymatically (Ebune et al., 1995). Upon hydrolysis of phytic acid, phosphorus is freed and the bioavailability of nutrients increases. Phytase is a phosphomonoesterase and is capable of hydrolyzing phytic acid to inorganic orthophosphate, lower esters of myoinositol, and free inositol (Irvine and Cosgrove, 1972). This enzyme is present in plants and tissues, and it is also produced by many species of fungi and bacteria (Consgrove, 1966). Supplementation of microbial phytases in animal diets provides growth performance equivalent to or better than animals supplemented with phosphate, and it reduces the amount of phosphorus in the animal manure (Wodzinski and Ullah, 1996). The U.S. Food and Drug Administration (FDA) has approved a generally–regarded–as–safe (GRAS) petition for use of phytase in food, and phytase has been marketed as a feed additive in the U.S. since 1996 (Wodzinski and Ullah, 1996).

Microbial enzymes are produced commercially mainly by submerged fermentation (SmF). However, the high cost of enzymes is often cited as the limiting factor prohibiting use of enzymes in animal diets (Han et al., 1987; Wodzinski and Ullah, 1996). An alternative enzyme production method, solid–state cultivation (SSC) has been reported to be a less expensive production method than SmF because it requires lower capital investment, has lower operating costs, and results in a higher volumetric productivity than SmF (Mitchell and Lonsane, 1992). SSC refers to the growth of microorganisms on solid substrates without the presence of free liquid (Cannel and Moo–Young, 1980). SSC is especially attractive for enzyme production for the animal feed industry because the entire fermented product can be dried, ground, and sold as animal feed, resulting in less waste and less downstream processing.

Environmental temperature of the fermenting solids is a significant variable in solid–state cultivation and is generally specific to the organism and the product to be cultured, as in submerged fermentations (Prior et al., 1992). Optimal temperatures for growth may not be the same as for product formation, suggesting a possible need for temperature shifts (profiling) in later stages of fermentation (Prior et al., 1992).

Previous phytase production research with *Aspergillus niger* focused on the effects of inoculum size (Krishna and Nokes, 2001), medium viscosity, and agitation levels (Papagianni et al., 2001). The influence of water content on the physical properties of the substrate and the growth of the microorganism is not well understood. Therefore, the optimum moisture content for each microbe–substrate system should be determined based on the desired product and the conditions for cultivation (Prior et al., 1992). The optimal moisture level is affected by temperature (Kim et al., 1985; Silman et al., 1979) and may not be the same for growth as it is for product formation (Grajek and Gervais, 1987).

The objective of this study was to investigate whether the phytase activity production coincides with fungal growth such that the optimal temperature and initial substrate water content for *Aspergillus niger* biomass growth would be the same for optimal phytase activity production. The median temperature and initial substrate moisture content values in this study were indicated by the producers of *Aspergillus niger* as being the best conditions for fungal growth. The upper and lower limits for temperature and initial substrate were selected to be different enough from the median values to elicit a change in the outcome, but within the limits of the specifications given by the supplier. Values outside of the upper and lower limits of this study would most likely result in subpar fungal growth such that phytase activity production would also be expected to be subpar. For example, we assumed that initial moisture contents greater than 65% would lead to anaerobic environment, which inhibits growth. In addition, we investigated the magnitude of substrate temperature and water content changes over time during SSC.

MATERIALS AND METHODS

ORGANISM AND MAINTENANCE

A phytase–producing strain of *Aspergillus niger* (provided by Alltech, Inc., Nicholasville, Ky.) was used throughout this work. Culture maintenance included a bimonthly recycle subculture from a molasses agar plate to a potato dextrose agar (PDA) plate and back, all stored at room temperature (~25°C). Inoculation was accomplished by cutting an agar block of $5 \times 5 \times 5$ mm from the growing edge of the culture and transferring it to the center of a new plate. The PDA plates were incubated for 7 days and used as the inoculum for the liquid culture. The inocula (a $5 \times 5 \times 5$ mm agar cube) for the solid–state culture were provided by a 3–day liquid shaker flask culture. The liquid media contained: 28 g/L corn starch, 5 g/L glucose, 18 g/L peptone, 0.5 g/L KCl, 1.5 g/L MgSO$_4$·7H$_2$O, 1 g/L KH$_2$PO$_4$, 2 g/L CaCl$_2$·2H$_2$O, and 20 g/L wheat bran. Corn starch was purchased from a local grocery store (Kroger Co.), wheat bran was purchased from a local organic food store (Good Foods Co–op), and all other reagents were purchased from Difco (Franklin Lakes, N.J.)

SOLID–STATE CULTIVATION

Solid–state cultivations of *Aspergillus niger* on a wheat bran and soybean meal substrate were conducted at three temperatures (25°C, 30°C, and 35°C) and three initial moisture contents (50%, 55%, and 60% w.b.) in a split–plot full–factorial experimental design with three replications. Fermentations were conducted for 0, 24, 48, 72, and 120 h. The containers were sampled destructively and assayed for phytase activity and for glucosamine as an estimate of biomass.

Glass 250 mL Erlenmeyer flasks were used as bench–scale bioreactors. The solid substrate, 3.5 g of wheat bran and 1.5 g of full–fat soybean meal (Good Foods Co–op, Lexington, Ky.), was added to each flask, and each flask was plugged with a foam stopper. Each flask was then equipped with a type–T thermocouple, supported by a glass rod, threaded through the foam stopper and positioned in the center of the solid substrate. All monitoring equipment and bioreactor parts were autoclaved at 121°C for 30 min prior to each experiment. Flasks were then inoculated aseptically with 3 mL of the inoculum solution containing mycelium, and the initial moisture content was adjusted using deionized water (50%, 55%, and 60% w.b.). Flasks were randomly placed in separate incubators (New Brunswick 4300, New Brunswick, N.J.) at three different temperatures (25°C, 30°C, and 35°C). Temperature readings were collected every 15 min with a datalogger (21X, Campbell Scientific, Inc., Logan, Utah). Cultures were harvested after 0, 24, 48, 72, and 120 h of fermentation.

PHYTASE ACTIVITY

Phytase activity was determine for the inoculum before cultivation and for the harvested phytase extracted from the solid substrate after cultivation. Crude enzyme was extracted using 20 mL of deionized water and 0.1% (v/v) Tween 80 per 1 g of initial media by homogenizing and shaking the suspension at 200 rpm for 1 h. The homogenized suspension was filtered through paper filters (Whatman 4, 15 cm), and the clear filtrate was designated as the crude enzyme. Phytase activity was assayed by measuring the amount of phosphorous released from a sodium phytate solution using the method of Harland and Harland (1980). The colorimetric analysis was performed with a spectrometer at 380 nm and compared to a standard curve. Enzyme activity was expressed in international units (IU) defined using the following equations:

$$\frac{\mu m}{mL\ enzyme} = \tag{1}$$

$$\left[\left(absorbance - blank@380\ nm\right)\left(standard\ curve\ slope\right)\right]\left(dilution\ factor\right)$$

$$\frac{IU}{g\ substrate} = \frac{\mu m}{mL\ enzyme}\ \frac{1}{time\ of\ reaction}\ \frac{100\ mL}{5\ g\ substrate} \tag{2}$$

One unit of enzyme activity was defined as the amount of phytase required to release 1 mg of phosphorus from 1 mL of 1.5 mM sodium phytate (pH of 4) per hour at room temperature.

FUNGAL BIOMASS ESTIMATION

After harvesting, 0.5 g of the SSC material was used for biomass estimation via a glucosamine assay, as described by

Sakurai et al. (1977). Glucosamine suspended in solution was quantified by a spectrophotometer at 530 nm. In order to estimate biomass based on glucosamine content, a calibration curve was generated by measuring the biomass and glucosamine contents of several submerged fermentations (Al–Asheh and Duvnjak, 1995).

EXPERIMENTAL DESIGN AND DATA ANALYSIS

A split–plot experiment incorporating factorial treatments was conducted to investigate the effects of environmental temperature, initial substrate moisture content, and length of fermentation on *Aspergillus niger* growth and phytase activity production with three replications (fig. 1). The main plot parameter was environmental temperature, due to the limited number of environmental chambers available. PROC GLM in SAS Software (SAS Institute, Inc., Cary, N.C.) was used to evaluate the data, using temperature within chamber mean square error as the error term for evaluating temperature main effects, and the model mean square error as the error term for evaluating substrate water content main effects and interactions ($\alpha = 0.05$, unless otherwise indicated).

RESULTS AND DISCUSSION

SUBSTRATE TEMPERATURE AND MOISTURE CONTENT DURING SSC

Substrate temperature achieved equilibrium with the environmental chamber within an hour of being placed in the chamber. The flasks cultivated in the 25°C environmental chamber maintained a temperature identical to the environmental chamber for approximately 50 h, then exhibited a small temperature increase (approximately 1°C over 20 h), and then remained fairly stable at 26°C until 95 h of cultivation, when the temperature returned to 25°C. The flasks in the 30°C environmental chamber exhibited a similar temperature trend, except the temperature increase began at approximately 40 h of fermentation, and the temperature reduction began at around 60 h of cultivation. The 35°C

flasks exhibited a larger temperature increase (approximately 2°C over 30 h), which began at approximately 25 h of cultivation. The temperature reduction began at around 50 h of fermentation, and it took around 40 h for the substrate temperature to return to 30°C.

Substrate moisture contents remained generally stable until 72 h of cultivation. The 50% w.b. moisture content treatment incubated at 25°C dried to about 28% w.b. by 120 h of fermentation. The 55% w.b. moisture content treatment incubated at 35°C dried to around 45% water content (w.b.) by 120 h. All other treatments remained within 5% of their initial water content throughout the fermentation.

PHYTASE ACTIVITY PRODUCTION

Average phytase activity results are shown in figure 2. For the 72 and 120 h fermentations, the main effect of temperature was significant according to the analysis of variance ($P < 0.01$), while the effect of moisture content and the interactions of the two were not significant.

For the 72 h fermentations, as shown in table 1, the main effects of temperature (averaged over initial moisture content) were significantly different for all three temperatures. The main effects of initial moisture content (averaged over temperature) were not significantly different. The only statistical difference between initial moisture content treatments within a single temperature treatment was between 50% and 60% initial moisture at 30°C (552 and 703 IU/g substrate, respectively). The highest level of phytase activity (703 IU/g substrate) was produced at 30°C with an initial moisture content of 60%.

After 120 h of fermentation, as shown in table 2, no main effect of initial moisture content was detected, while the phytase activity for the 30°C temperature (averaged over initial moisture content) of 740 IU/ g substrate was statistically higher than for the other temperature treatments. With respect to the treatment combinations, the only significant difference was seen with an initial moisture content of 50%, where the phytase activity at 30°C (790 IU/g substrate) was statistically higher than the phytase activity at the other temperatures (477 IU/g substrate at 25°C and 343 IU/g substrate at 35°C).

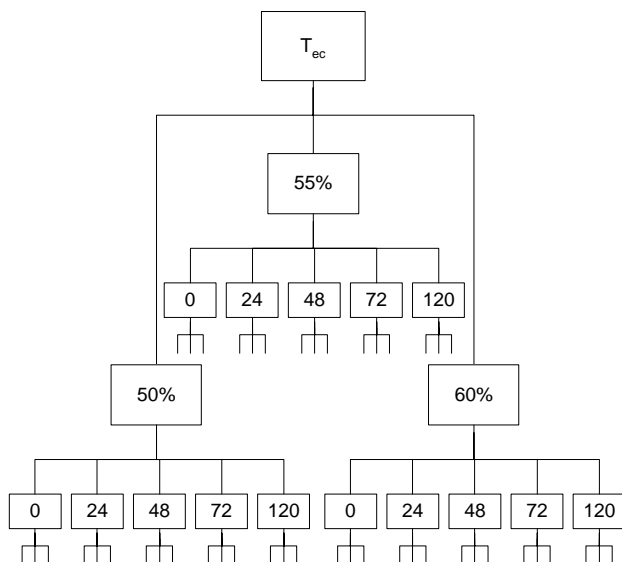**Figure 1. Diagram of a single main plot (temperature) with three split–plot treatments (initial moisture content), five fermentation times, and three replications for each treatment combination.**
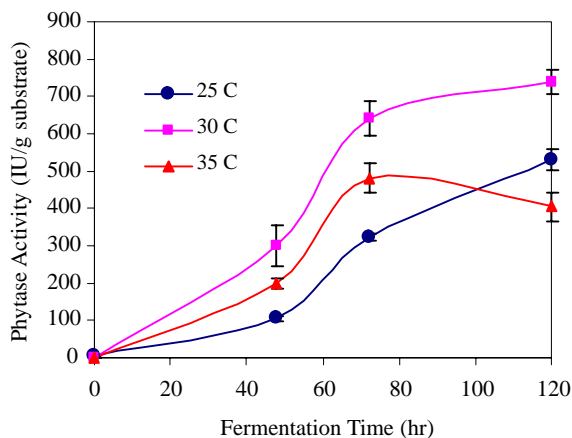

Figure 2. The increase in phytase activity during fermentation averaged over moisture content for each temperature treatment (average of three moisture levels and three replications). Error bars represent standard error.

Table 1. Phytase activity (IU/g substrate) after 72 h of fermentation for all temperature (°C) and initial moisture content (% w.b.) treatment combinations.[a]

Temperature (°C)	Initial Moisture Content			Temperature Main Effect Average (IU/g substrate)
	50%	55%	60%	
25	316 ad	312 ad	343 ad	324 a
30	552 bd	671 bde	703 be	642 b
35	413 ad	548 bd	484 ad	482 c
Moisture content main effect average (IU/g substrate)	427 d	510 d	510 d	

[a] Different letters (a, b, and c) indicate different column means for temperature treatments within each moisture content; different letters (d, e, and f) indicate different row means for moisture content treatments within each temperature treatment (α = 0.05).

FUNGAL GROWTH

Average glucosamine production for the three temperature levels (averaged over moisture content) is shown in figure 3. For the 72 and 120 h fermentations, the effect of temperature on glucosamine production was significant according to the analysis of variance (P < 0.005), while the effect of moisture content and the interactions of the two were only significant for the 120 h fermentations (P < 0.05).

After 72 h of fermentation, as shown in table 3, the main effects of temperature (averaged over initial moisture content) were significantly different for all three temperatures, with the 35°C treatment resulting in the highest glucosamine production (19.80 mg/g). The 30°C temperature environment produced the second highest biomass glucosamine level (15.34 mg/g). The first observation of differences in biomass glucosamine production by moisture content occurred at 72 h of fermentation. Biomass glucosamine production at 50% initial moisture content (12.97 mg/g) was significantly lower than production at 55% moisture content (15.10 mg/g), when averaged over temperature. The highest accumulation of biomass glucosamine (18.90 mg/g) occurred with a temperature of 35°C and a 60% initial moisture content, a noticeable contrast from the 48 h fermentation data where it ranked the lowest among moisture contents at a temperature of 35°C.

After 120 h of fermentation, as shown in table 4, the main effect of temperature was significantly different for all temperatures averaged over initial moisture content. The 35°C treatment resulted in the highest glucosamine (24.16 mg/g), followed by the 30°C treatment (16.15 mg/g)

Table 2. Phytase activity (IU/g substrate) after 120 h of fermentation for all temperature (°C) and initial moisture content (% wet basis) treatment combinations.[a]

Temperature (°C)	Initial Moisture Content			Temperature Main Effect Average (IU/g substrate)
	50%	55%	60%	
25	477 ad	555 ad	562 ad	531 a
30	790 bd	679 ad	752 ad	740 b
35	343 ad	394 ad	476 ad	404 a
Moisture content main effect average (IU/g substrate)	537 d	543 d	597 d	

[a] Different letters (a, b, and c) indicate different column means for temperature treatments within each moisture content; different letters (d, e, and f) indicate different row means for moisture content treatments within each temperature treatment (α = 0.05).
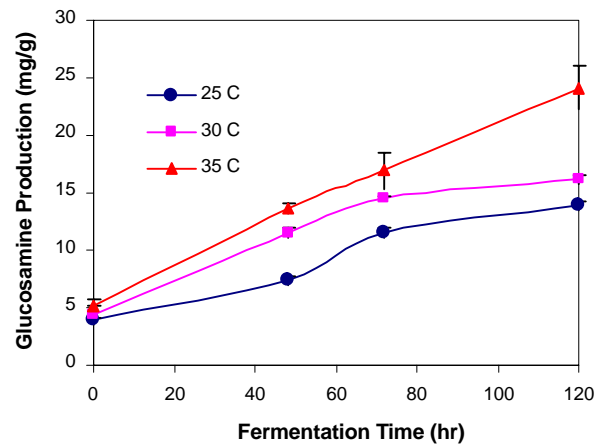

Figure 3. The increase glucosamine during fermentation averaged over moisture content for each temperature treatment (average of three moisture levels and three replications). Error bars represent standard error.

and then the 25°C treatment (13.92 mg/g). A significant effect of initial moisture content averaged over temperature was observed, where the 50% and 60% initial moisture contents resulted in statistically higher fungal growth (18.34 and 19.19 mg/g, respectively). For the 55% initial moisture content, the higher temperatures corresponded to a decrease in fungal growth compared to the other initial moisture treatments, where this initial moisture content resulted in an increase in fungal growth at 72 h of fermentation. This seems to indicate that the fungal growth peaks earlier than 120 h.

Table 3. Glucosamine production (mg/g) activity after 72 h of fermentation for all temperature (°C) and initial moisture content (% wet basis) treatment combinations.[a]

Temperature (°C)	Initial Moisture Content			Temperature Main Effect Average (mg/g)
	50%	55%	60%	
25	10.62 ad	12.25 ad	11.60 ad	11.49 a
30	14.60 bd	14.87 abd	14.19 ad	15.34 b
35	13.68 abd	18.18 be	18.90 be	19.80 c
Moisture content main effect average (mg/g)	12.97 d	15.10 e	14.90 de	

[a] Different letters (a, b, and c) indicate different column means for temperature treatments within each moisture content; different letters (d, e, and f) indicate different row means for moisture content treatments within each temperature treatment (α = 0.05).

Table 4. Glucosamine production (mg/g) activity after 120 h of fermentation for all temperature (°C) and initial moisture content (% wet basis) treatment combinations.[a]

Temperature (°C)	Initial Moisture Content			Temperature Main Effect Average (mg/g)
	50%	55%	60%	
25	14.70 ad	13.55 ad	13.51 ad	13.92 a
30	16.57 ad	15.33 ad	16.54 bd	16.15 b
35	23.75 bd	21.21 bd	27.53 ce	24.16 c
Moisture content main effect average (mg/g)	18.34 de	16.70 d	19.19 e	

[a] Different letters (a, b, and c) indicate different column means for temperature treatments within each moisture content; different letters (d, e, and f) indicate different row means for moisture content treatments within each temperature treatment (α = 0.05).

CONCLUSION

The maximum phytase activity was produced at 30˚C and 55% to 60% initial substrate moisture content for a 72 h fermentation and at 30˚C and 50% to 60% moisture content for a 120 h fermentation. The change in phytase activity from the 72 h (30˚C; 55% and 60% moisture contents) fermentation to the 120 h (30˚C; 50%, 55%, and 60% moisture contents) was not statistically significant ($\alpha = 0.05$). The glucosamine quantities produced under both sets of conditions were compared statistically to determine if they differed, but no statistical difference was found. The shorter fermentation time would allow a higher production rate in an industrial enzyme production plant.

The maximum fungal growth was produced at 35˚C, either 50% or 60% initial substrate moisture content, and 120 h of fermentation, and was statistically higher than under similar conditions at 72 h of fermentation. The higher temperature required for optimal growth, as opposed to optimal phytase productions, indicates that the production of phytase is not directly dependent on fungal growth. Environmental temperature affected phytase activity production, but substrate initial moisture content had less of an effect. The results of this study suggest that a lower temperature (30˚C) should be used to maximize phytase production. A shorter fermentation time (72 h) should be sufficient for phytase production. With a shorter fermentation time, the moisture content should be between 55% and 60%.

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