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Optimization of phytase production by solid substrate fermentation

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Abstract The production of phytase by three feed-grade filamentous fungi (*Aspergillus ficuum* NRRL 3135, *Mucor racemosus* NRRL 1994 and *Rhizopus oligosporus* NRRL 5905) on four commonly used natural feed ingredients (canola meal, cracked corn, soybean meal, wheat bran) was studied in solid substrate fermentation (SSF). *A. ficuum* NRRL 3135 had the highest yield [15 IU phytase activity/g dry matter (DM)] on wheat bran. By optimizing the supplementation of wheat bran with starch and $(NH_4)_2SO_4$, phytase production increased to 25 IU/g DM. Optimization was carried out by Plackett-Burman and central composite experimental designs. Using optimized medium, phytase, phosphatase, alpha-amylase and xylanase production by *A. ficuum* NRRL 3135 was studied in Erlenmeyer flask and tray SSF. By scaling up SSF from flasks to stationary trays, activities of 20 IU phytase activity/g DM were reproducibly obtained.

Keywords Phytase · Solid substrate fermentation · *Aspergillus ficuum* · Optimization · Statistical experimental design

Introduction

An indispensable requirement for sustainable agriculture is the reduction of the environmental burden caused by agricultural practices. Large-scale animal production (pig and poultry farms and cattle feedlots) generates enormous quantities of potentially hazardous waste. Phosphorous (P) is an important pollutant in animal waste. In grains, roughly 60–80% of P is tied up in phytin, an organic acid that is not digestible by monogastric animals. Ruminant animals, such as cattle, sheep, and goats, appear able to digest phytin only partially. The phytin thus winds up in manure and liquid effluent and can be degraded by aquatic microorganisms, which causes environmental pollution. In the European Union, laws limit the P content in animal waste, and stiff penalties are set for exceeding that limit. The United States will shortly introduce such regulations, and other countries and regions are also expected to do so [16, 25]. These regulations prompt farmers to reduce P pollution by adding phytase to monogastric animal feed.

Phytase is an enzyme that makes the P from phytin available for animal digestion [21]. Reduction or elimination of inorganic phosphate supplementation to animal feed reduces P in the manure by about 33%, thus cutting the pollution burden by one-third. The drawback to the widespread application of phytase as a feed supplement is the current high price of commercial phytase, which may add US $2–3 per metric ton to the feed cost [4]. The enzyme is currently produced by conventional submerged fermentation (SF), a more expensive high technology process.

An economical alternative for enzyme production and application would be solid substrate fermentation (SSF) [13, 20, 22, 32]. Many enzymes and other biochemicals can be produced by SSF at a fraction of the cost of SF production [29, 31]. Phytase may be produced directly in SSF by filamentous fungi on selected feed ingredients, and the crude product may be mixed in feed rations as a value-added supplement. The fungal product contains not only phytase, but also accessory enzymes, cellulases, hemicellulases, pectinases, amylases, fungal protein and organic acids that increase feed digestibility and access to phytin in plant cells [8, 9, 20, 22,

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23]. Phytase production by SSF is described in the literature without any rigorous studies on optimizing fermentation conditions for maximal phytase production [8, 9, 20, 21, 23, 26, 27]. Filamentous fungi of the genera *Aspergillus*, *Mucor* and *Rhizopus* are the best producers in SSF.

In this paper, SSF optimization and scale-up of phytase production by selected fungi on selected substrates are described.

Materials and methods

Fungi and inoculum preparation

Aspergillus ficuum NRRL 3135, *Mucor racemosus* NRRL 1994 and *Rhizopus oligosporus* NRRL 5905 strains were obtained from the Northern Regional Research Center, (USDA, Peoria, Ill.). Strains NRRL 1994 and NRRL 5905 are recommended to produce the fermented foods sufu and tempeh, respectively. The fungi were grown and maintained on potato dextrose agar (PDA) slants. Viable spores from 6-day-old fully sporulated slants were harvested by washing them with 0.1% Tween-80-containing water, and the spore suspension was adjusted to 10^6 cfu (colony forming units on PDA plates) per milliliter for inoculation.

Substrates

The substrates were obtained from the following companies: defatted canola meal (Archer Daniels Midland, Velva, N.D.); corn meal (local market); defatted soybean meal (Soybean Processors, Volga, S.D.) and wheat bran (Cortez Milling Company, Cortez, Colo.). Molasses was a gift from the Western Sugar Company (Fort Morgan, Colo.). Corn steep liquor was purchased from Sigma (St. Louis, Mo.).

SSF in Erlenmeyer flasks

Air-dried substrate (10 g) was placed in 500 ml cotton-plugged Erlenmeyer flasks, supplemented with 5 ml salt solution containing (g/l): NH_4NO_3, 23; $MgSO_4{\cdot}7H_2O$, 4.6; NaCl, 4.6, and different moisture levels were adjusted with tap water. The wet substrate was sterilized at 121°C for 20 min. After it had cooled the substrate was inoculated with 1 ml spore suspension of the respective fungus at 10^6 cfu/ml. The flasks were incubated for 3 days (*M. racemosus* and *R. oligosporus*), or 4 days (*A. ficuum*) at 26 ± 1°C, unless otherwise noted. All experiments were carried out in duplicate. The results shown are average values.

SSF in trays

Air-dried wheat bran (1,500 g) was wetted with 2.25 l of a solution containing 20 g/l starch and 17 g/l $(NH_4)_2SO_4$, sterilized at 121°C for 60 min. The cooled substrate was inoculated with 150 ml spore suspension of *A. ficuum* NRRL 3135 containing 10^6 cfu/ml. The moisture content of the inoculated substrate was 62%. The inoculated substrate was spread on a separately sterilized aluminum tray (780×510×80 mm) to about 3 cm thick, covered with two layers of plastic wrap, and incubated for 5 days at 26 ± 1°C in a 99% relative humidity chamber. The trays were ventilated twice each day for a few minutes. Samples were taken aseptically each day from different parts of the trays to obtain representative composite samples.

Analytical methods

Enzyme activities were determined from the culture extracts (SSF). SSF samples were extracted with water containing 0.1% Tween-80 (10 ml water/g dry substrate), by shaking them for 1 h at 200 rpm at room temperature (~25°C). The suspension was centrifuged (10,000 *g*, 10 min) and the supernatant was stored at 4°C until assays were performed.

Alpha-amylase activity was determined as described by Okolo et al. [19]. The reaction mixture consisted of 1.25 ml 1% soluble starch (Merck, Darmstadt, Germany) solution, 0.25 ml 0.1 M sodium acetate buffer (pH 5.0), 0.25 ml distilled water and 0.25 ml appropriately diluted crude enzyme solution. After 10 min incubation at 50°C, the liberated reducing sugars (glucose equivalent) were estimated by the dinitrosalicylic acid method of Miller [17]. One unit of alpha-amylase is defined as the amount of enzyme releasing 1 μmol glucose equivalent per minute under the assay conditions.

Phytase activity was assayed by measuring the inorganic phosphorus released from sodium phytate solution using the method described by Harland and Harland [11]. The reaction mixture consisted of 1 ml 0.1 M $MgSO_4{\cdot}7H_2O$, 2.4 ml 6.82 mM phytic acid and 0.6 ml appropriately diluted crude enzyme solution. The $MgSO_4{\cdot}7H_2O$ and phytic acid solutions were prepared with 0.2 M sodium acetate buffer (pH 5.15). The reactions were carried out at 55°C for 60 min, and were stopped by adding 0.5 ml 10% trichloroacetic acid. The blue color was developed by adding first 1 ml water and then 2.5 ml Taussky-Schoor reagent. After the contents were mixed, the absorbance was read immediately at 660 nm. One unit of phytase is defined as the amount of enzyme releasing 1 μmol inorganic P per minute under the assay conditions.

Phosphatase activity against 4-nitrophenyl phosphate was determined by incubating 1 ml of properly diluted crude enzyme solution with an equal volume of 0.64% 4-nitrophenyl phosphate disodium salt dissolved in 0.2 M sodium acetate buffer (pH 5.15) [24]. After 15 min of incubation at 37°C the reaction was terminated by cooling. The color developed by the liberated *p*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of phosphatase is defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute under the assay conditions.

Xylanase activity was assayed using a 1% solution of xylan (4-*O*-methyl glucuronoxylan from birchwood; Sigma) as a substrate [3]. The release of reducing sugars in 10 min at 50°C was measured as xylose using the dinitrosalicylic acid method [17]. One unit of xylanase is defined as the amount of enzyme releasing 1 μmol xylose equivalent per minute under the assay conditions.

Soluble protein was determined spectrophotometrically according to the method described by Lowry et al. [14].

Optimization studies and statistical analysis

Plackett-Burman screening designs allow the testing of multiple independent variables within a single experiment [28]. After the independent variables and their corresponding levels have been selected, the experiments are performed and the responses, such as enzyme production, are measured. These experiments were carried out in flasks using 10 g air-dried wheat bran plus 11 medium ingredients, including 3 carbon sources, 3 organic nitrogen sources, 4 inorganic nitrogen sources and 1 inorganic phosphate source, under the conditions described above at constant 60% moisture content. The effect of each variable upon the measured response was determined by the difference between the average of the + and − responses. The significance level of the effect of each variable was determined by Student's *t* test. The effect of a factor is considered to be significant, if $t_{\alpha/2} < t(12)$. $t_{\alpha/2} = 2.179$ at the probability level of $\alpha \leq 0.05$ and $df = 12$.

Central composite design was carried out to determine the optimal concentrations of the two variables previously selected by Plackett-Burman design [6, 10]. Using this design, each factor can

be set at five levels, coded −a, −1, 0, +1, +a. Optimizing two factors includes a full 2^2 factorial design with four possible combinations of the two factors at level −1 or +1 augmented with two replications of the center point and the four star points. Star points have an axial distance to the center of ±a for one factor, whereas the other factor is at level 0. The axial distance "a" was chosen to be 1.414 to make this design rotatable.

Statistica for Windows (StatSoft, Bedford, UK) was used for regression analysis of the experimental data obtained. To determine the significance of the regression coefficients, a *t*-test was applied. All experimental designs were randomized. Experiments were performed in duplicate in flasks and the average values are shown.

Results and discussion

Phytase production on common feed ingredients

Phytase was produced by three feed-grade filamentous fungi on four commonly used natural feed ingredients. The phytase activities obtained at the optimum moisture level for each substrate are shown in Table 1. *A. ficuum* NRRL 3135 gave the highest yield [15.29 IU phytase activity/g dry matter (DM)] at 60% moisture level on wheat bran. Moisture content of the medium in SSF is very important for the growth of microorganisms, production of enzymes and for enzyme activity. The effect of moisture content on phytase production by *A. ficuum*, *M. racemosus* and *R. oligosporus*, in four different substrates (canola meal, cracked corn, soybean meal, wheat bran) was compared in SSF as described in Materials and methods, using three different moisture levels (Fig. 1). Moisture content influenced phytase production significantly. Each substrate and each fungus had its own optimal moisture content for phytase production. With *A. ficuum* the best relative growth and phytase production were observed on wheat bran. *R. oligosporus* grew well on wheat bran but produced phytase only on canola meal. The phytase-producing potential of various filamentous fungi by SSF has been examined in more detail elsewhere [5]. Among fungi examined, *M. racemosus* NRRL 1994 produced the best result, 26 IU phytase activity/g DM on optimized coconut press cake medium [5]. Others have reported good growth and phytase production on canola meal, and on a mixture of wheat bran and soybean meal [1, 8, 15, 23].

Optimization of medium composition for SSF by *A. ficuum*

The chemical composition of the different feed ingredients, the quality of the carbon and nitrogen sources and the phosphate content may regulate phytase production. In a preliminary study, it was found that, at equivalent nitrogen levels, ammonium sulfate and ammonium chloride were better than ammonium nitrate or other natural nitrogen sources for phytase production by *A. ficuum* NRRL 3135 (data not shown). The importance of the quality of the nitrogen source was also observed in cellulase and xylanase production by SSF, possibly by affecting pH regulation [29, 30]. In another preliminary study it was found that a KH_2PO_4 level above 10 mg/g DM substrate inhibited phytase production by *A. ficuum* NRRL 3135 in SSF (data not shown). Phosphate inhibition of phytase production in SSF was also observed for *M. racemosus* NRRL 1994 [5] and this has been corroborated by other researchers for *A. ficuum* [8] and *A. carbonarius* [2].

In a systematic study aimed at optimizing phytase production by *A. ficuum* NRRL 3135 in SSF, wheat bran was supplemented with 11 ingredients that are very likely to influence enzyme production. These include carbohydrates, nitrogen sources and phosphate. The effect of the selected 11 ingredients was studied in an 11-factor Plackett-Burman design (Table 2). The regression coefficients and *t*-values for the 11 ingredients are presented in Table 3. The coefficient of determination, r^2, was calculated to be 0.96. This indicates that the model explains 96% of the variability in the data. Starch, urea and ammonium salts constituted positive effects on the production of phytase by the fungal culture. Based on these results, starch and ammonium sulfate were chosen for determination of their optimal concentrations in the SSF medium by central

Table 1 Comparison of relative growth (*X*) and production of phytase activity (*PA*) by three filamentous fungi on natural feeding redients in solid substrate fermentation (SSF). Fermentation conditions:10 g air-dried substrate in a 500 ml Erlenmeyer flask was supplemented with 5 ml salt solution containing (g/l) NH_4NO_3, 23; $MgSO_4{\cdot}7H_2O$, 4.6; NaCl, 4.6; different moisture levels were adjusted by adding water; inoculum: 10^5 spores/g substrate; incubation temperature: 26 ± 1°C; fermentation time: 3 days for *Mucor racemosus* and *Rhizopus oligosporus* and 4 days for *Aspergillus ficuum*. Optimal moisture content for each substrate is shown in parentheses

Strain	Substrate							
	Canola meal		Corn meal		Soybean meal		Wheat bran	
	X[a]	PA[b]	X	PA	X	PA	X	PA
A. ficuum NRRL 3135	+ + +	5.73 (60%)	+ +	1.94 (60%)	+ +	7.18 (60%)	+ + + +	15.29 (60%)
M. racemosus NRRL 1994	+ + +	5.78 (70%)	+ + +	0.51 (60%)	+ + +	1.36 (70%)	+ + + + +	7.91 (75%)
R. oligosporus NRRL 5905	+ +	6.31 (70%)	+ + +	0.10 (60%)	+	1.36 (70%)	+ + + + +	0.78 (70%)

[a]Intensity of growth by visual observation
[b]IU/g dry matter (DM)

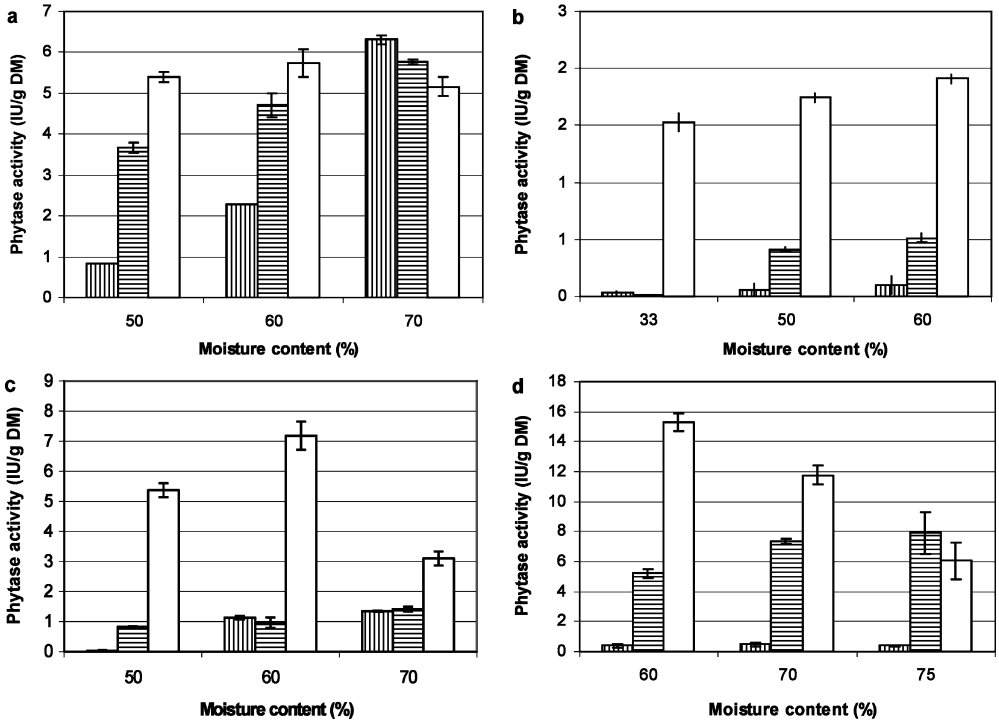

Fig. 1a–d Effect of moisture content on phytase production by three filamentous fungi in solid substrate fermentation on natural feed ingredients. Fermentation conditions: 10 g air-dried substrate in a 500 ml Erlenmeyer flask was supplemented with 5 ml salt solution containing (g/l) NH_4NO_3, 23; $MgSO_4 \cdot 7H_2O$, 4.6; NaCl, 4.6 and different moisture levels were adjusted by adding water; inoculum: 10^5 spores/g substrate; incubation temperature: $26 \pm 1°C$; fermentation time: 3 days. *Mucor racemosus* NRRL 1994 (*horizontally shaded bars*), *Rhizopus oligosporus* NRRL 5905 (*vertically shaded bars*) and 4 days *Aspergillus ficuum* NRRL 3135 (*white bars*). **a** Defatted canola meal, **b** corn meal, **c** defatted soybean meal, **d** wheat bran

Table 2 Plackett-Burman design for 11 wheat bran supplements, assigned concentrations of factors at different levels and the experimental results. Fermentation conditions: 10 g air-dried wheat bran and different additives; moisture level: 60%; inoculum: 10^5 spores of *A. ficuum* NRRL 3135/g substrate; incubation temperature: $26 \pm 1°C$; fermentation time: 4 days

Trial no.	Factors and factor levels (g/g dry substrate %)											Phytase activity (IU/g DM)
	Starch	Glucose	Molasses	Corn steep liquor	Soybean meal	Urea	NH_4NO_3	NH_4Cl	$(NH_4)_2SO_4$	KNO_3	KH_2PO_4	
1	4.5	0.45	9.0	0	0	0	0.56	0.75	0.93	0	0.75	17.7
2	4.5	4.5	0.9	12	0	0	0	0.75	0.93	1.41	0	12.8
3	0.45	4.5	9.0	0	6.4	0	0	0	0.93	1.41	0.75	6.9
4	4.5	0.45	9.0	12	0	0.43	0	0	0	1.41	0.75	9.0
5	4.5	4.5	0.9	12	6.4	0	0.56	0	0	0	0.75	11.0
6	4.5	4.5	9.0	0	6.4	0.43	0	0.75	0	0	0	11.6
7	0.45	4.5	9.0	12	0	0.43	0.56	0	0.93	0	0	14.0
8	0.45	0.45	9.0	12	6.4	0	0.56	0.75	0	1.41	0	10.4
9	0.45	0.45	0.9	12	6.4	0.43	0	0.75	0.93	0	0.75	15.5
10	4.5	0.45	0.9	0	6.4	0.43	0.56	0	0.93	1.41	0	14.1
11	0.45	4.5	0.9	0	0	0.43	0.56	0.75	0	1.41	0.75	11.7
12	0.45	0.45	0.9	0	0	0	0	0	0	0	0	10.3
13	2.48	2.48	4.95	6.0	3.2	0.21	0.28	0.38	0.47	0.71	0.38	13.6

composite design. The design of this experiment and the experimental results are shown in Table 4. Analysis of variance was performed in order to validate the regression model. The regression coefficients, *t*- and *P*-values are presented in Table 5. The fit of the model is expressed by r^2, which was calculated to be 0.88. The model therefore accounts for 88% of the response. The coordinates of the maximum point were found to be $A = -0.13$, $B = 0.16$, corresponding to the optimal supplementation levels for starch and ammonium sulfate of 3.0 and 2.55 g/100 g dry wheat bran, respectively (Fig. 2). In all further experiments wheat bran was supplemented with these optimized levels of starch and ammonium sulfate.

Table 3 Results of regression analysis for the Plackett-Burman design. Coefficient of determination, $r^2 = 0.96$

Term	Coefficient	*t*-value	*P*-value
Intercept	12.17	78.29	0.000
Starch	0.61	3.75	0.002
Glucose	−0.77	−4.74	0.000
Molasses	−0.48	−2.97	0.011
Corn steep liquor	−0.03	0.20	0.841
Soybean meal	−0.49	−3.06	0.009
Urea	0.56	3.47	0.004
NH_4NO_3	1.07	6.61	0.000
NH_4Cl	1.19	7.41	0.000
$(NH_4)_2SO_4$	1.4	8.69	0.000
KNO_3	−1.27	−7.90	0.000
KH_2PO_4	−0.11	−0.70	0.497

Table 4 Central composite design, assigned concentrations of starch and ammonium sulfate at different levels and the experimental results. Fermentation conditions: 10 g air-dried wheat bran and different additives; moisture level: 60%; inoculum: 10^5 spores of *A. ficuum* NRRL 3135/g substrate; incubation temperature:26 ± 1°C; fermentation time: 4 days

Trial no.	Levels		Phytase activity
	(g/g dry substrate %)		(IU/g DM)
	Starch (A)	$(NH_4)_2SO_4$ (B)	
1	1.3	0.9	16.7
2	1.3	3.9	17.5
3	5.1	0.9	15.0
4	5.1	3.9	18.3
5	0.5	2.4	22.9
6	5.9	2.4	20.7
7	3.2	0.3	13.1
8	3.2	4.5	18.6
9	3.2	2.4	25.1
10	3.2	2.4	22.6

Table 5 Results of regression analysis of the centralcomposite design. Coefficient of determination, $r^2 = 0.88$

Term	Coefficient	*t*-value	*P*-value
Intercept	23.83	26.58	0.000
A	−0.49	−1.10	0.288
B	1.49	−2.52	0.005
AB	0.62	0.98	0.344
A^2	−1.49	3.32	0.025
B^2	−4.46	−7.53	0.000

Estimation of the economy of phytase production by SSF

The kinetics of phytase production by laboratory scale SSF on the optimized wheat bran medium is shown in Fig. 3. Phytase activity reached 25 IU/g DM in 4 days, accompanied by significant activities of other hydrolytic enzymes, such as alpha-amylase, xylanase and phosphatase, necessary for the efficient degradation of phytin in plant cells.

In an attempt to approximate industrial conditions for phytase production, SSF was conducted in a stationary tray, similar to conditions in traditional Koji reactors [18]. In this way 3.75 kg crude SSF enzyme was produced in one batch in a tray, enough for feed evaluation. In this first attempt 20 IU phytase activity/g DM substrate was reached in 4 days, together with substantial levels of other hydrolytic enzymes (Fig. 4). These results are encouraging for optimization under pilot scale or industrial scale conditions.

The product of this fermentation is an enriched feed ingredient that contains phytase and hydrolytic enzymes that can be easily mixed with other ingredients in feed. The economy of the SSF enzyme appears to be favorable. Assuming a modest phytase production of 20 IU/g DM substrate, a recommended 400 IU/kg feed supplementation would require only 20 kg crude SSF enzyme (whole SSF culture) to be added to each metric ton (MT) of feed. By using an assumed fermentation cost of US $50/MT substrate (based on comparison with the costs of established SSF processes, composting about US $6/MT, ensiling about US $10/MT, malting about US $20/MT), the estimated cost could be an affordable US $1.00/MT feed. In the literature, phytase production by SF is in the range 0.1–15 IU/ml [7, 12, 23], corresponding to 2.0–300 IU/g DM substrate in a 5% slurry. These data are subject to a large degree of uncertainty, because different authors use different techniques for phytase determination. At such production levels the cost of the crude enzyme would be much higher than the cost of the SSF enzyme. To have the same economy as SSF, about 80 IU/ml SF production would be necessary to achieve the same US $1.00/MT enzyme cost. (assumption: 5 l crude enzyme/MT feed at 400 IU/kg feed level; at US $200/$m^3$ fermentation cost the 5 l

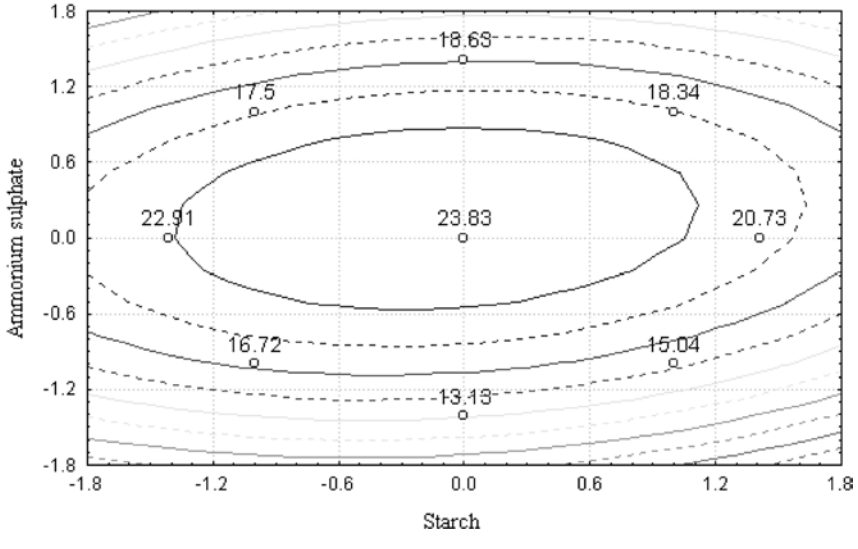

Fig. 2 Contour plot reflecting the effect of quantity of starch and ammonium sulfate on phytase production of *A. ficuum* NRRL 3135. Fermentation conditions: 10 g air-dried wheat bran supplemented with different concentration levels of starch and $(NH_4)_2SO_4$; moisture level: 60%; inoculum: 10^5 spores/g substrate; incubation temperature: 26 ± 1°C

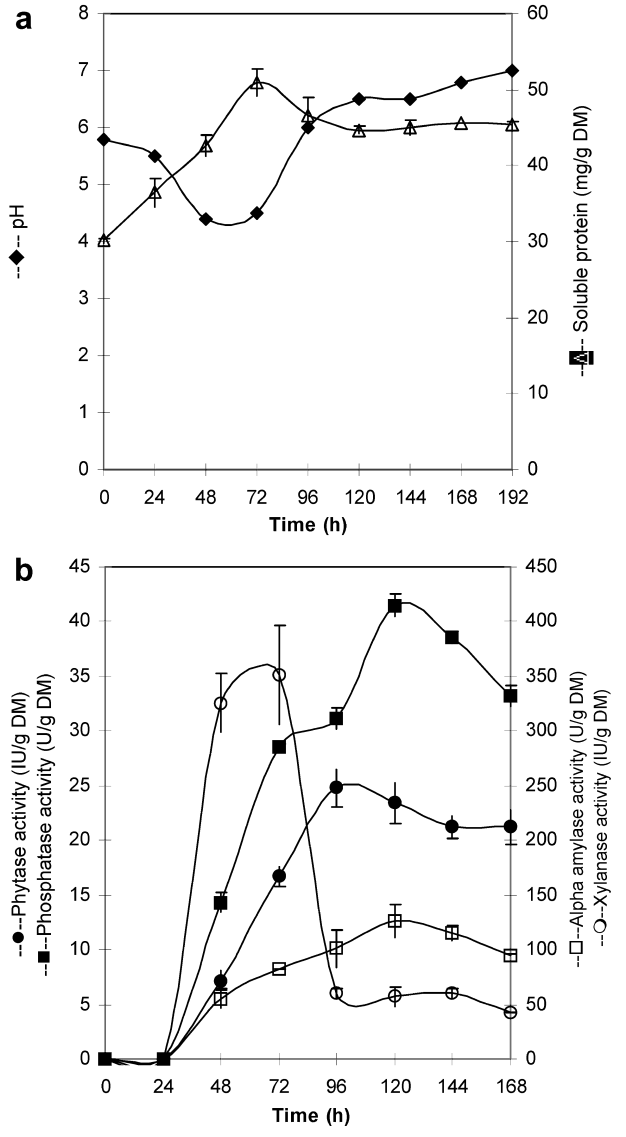

Fig. 3a, b Kinetics of solid substrate fermentation by *A. ficuum* NRRL 3135 in 500 ml Erlenmeyer flasks. **a** pH and soluble protein, **b** enzyme activities. Fermentation conditions: 10 g air-dried wheat bran was supplemented with 15 ml nutrient solution containing 20 g/l starch, 17 g/l $(NH_4)_2SO_4$; inoculum: 10^5 spores/g substrate; incubation temperature: $26 \pm 1°C$

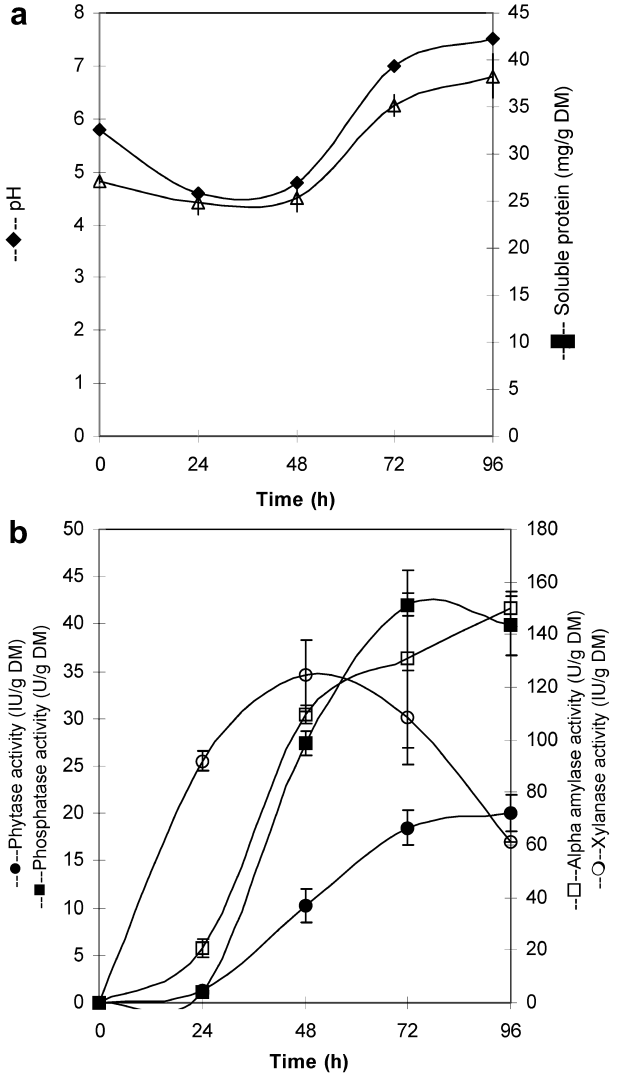

Fig. 4a, b Kinetics of solid substrate fermentation by *A. ficuum* NRRL 3135 in a tray (koji) bioreactor. **a** pH and soluble protein, **b** enzyme activities. Fermentation conditions: 1,500 g air-dried wheat bran on an aluminum tray ($780 \times 510 \times 80$ mm) was supplemented with 2.25l nutrient solution containing 20 g/l starch, 17 g/l $(NH_4)_2SO_4$; inoculum: 10^5 spores/g substrate; incubation temperature: $26 \pm 1°C$

enzyme would cost US $1.00). Such a high productivity may be achieved only with genetically improved strains. The reported results in SSF, however, were reached with a natural wild strain, thus the potential for genetic improvement is very high.

Conclusions

Phytase may be produced efficiently on natural feed ingredients by different filamentous fungi in SSF, most efficiently by *A. ficuum* on wheat bran. By nutrient optimization—selecting the most appropriate carbon and nitrogen sources—the production level was increased by 50%. Scale-up in simple tray reactors produced adequate results, but advanced bioreactors that provide accurate moisture and temperature control, as well as optimized O_2 mass transfer, promise further process improvement. SSF appears to be a more efficient, less costly, more directly applicable process than currently available SF technology. The accessory hydrolytic enzymes represent a bonus value, and greatly increase the feed value, digestibility and accessibility of plant phytin to phytase attack. The SSF enzyme would compete favorably with the best available commercial phytase preparations.

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