Production of Phytase by *Mucor racemosus* in Solid-State Fermentation

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Phytase production was studied by three *Mucor* and eight *Rhizopus* strains by solid-state fermentation (SSF) on three commonly used natural feed ingredients (canola meal, coconut oil cake, wheat bran). *Mucor racemosus* NRRL 1994 (ATCC 46129) gave the highest yield (14.5 IU/g dry matter phytase activity) on coconut oil cake. Optimizing the supplementation of coconut oil cake with glucose, casein and $(NH_4)_2SO_4$, phytase production in solid-state fermentation was increased to 26 IU/g dry matter (DM). Optimization was carried out by Plackett−Burman and central composite experimental designs. Using the optimized medium phytase, α-amylase and lipase production of *Mucor racemosus* NRRL 1994 was compared in solid-state fermentation and in shake flask (SF) fermentation. SSF yielded higher phytase activity than did SF based on mass of initial substrate. Because this particular isolate is a food-grade fungus that has been used for sufu fermentation in China, the whole SSF material (crude enzyme, in situ enzyme) may be used directly in animal feed rations with enhanced cost efficiency.

Introduction

The production of feed-grade enzymes such as amylases, cellulases and phytases by solid-state fermentation (SSF) and their application in animal feed rations have been studied in different laboratories (*1*−*10*). Phytases are key enzymes to supplement animal feed. Phytases (*myo*-inositol hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8. and *myo*-inositol hexakisphosphate 6-phosphorylase, EC 3.1.3.26.) are classified as the family of histidine acid phosphatases that catalyze the hydrolysis of phosphate from phytic acid to inorganic phosphate and *myo*-inositol phosphate derivatives. The released phosphorus can be used in metabolic processes. These enzymes are present in many plants and animal tissues and are also produced by many species of fungi and bacteria (*11*).

Mineral nutrients are stored in all seeds, most commonly as mixed cation salts of *myo*-inositolhexaphosphoric acid (phytic acid) (*12*). Making up about 1−8% of the dry weight of mature seeds, phytate accounts for as much as 90% of their total phosphorus content. However, monogastric animals, such as pigs, poultry and fish do not utilize this source of phosphate, lacking the requisite gastrointestinal tract enzyme(s) for release of the phosphate from phytate (*13*). Thus, producers are forced to employ expensive supplementary phosphorus to meet animal dietary requirements. Further, phytate binds to multivalent cations, thereby reducing the bioavailability of these ions. Phytate also inhibits enzymes, such as α-amylase, trypsin, tyrosinase and pepsin (*14*).

The catalyzed release of phosphate from phytate occurs naturally in soil and water environments, and thus phytate in manure can cause serious phosphorus pollution. In areas of the world where monogastric livestock production is intensive, this problem can make a significant contribution to the eutrophication of surface waters (*15*).

In the European Union, laws limit the phosphorus content in animal waste, and stiff penalties are set for exceeding that limit. The U.S. will shortly introduce such regulations, and other countries and regions are expected to do so (*16*, *17*). These regulations prompt farmers to reduce phosphorus pollution by adding phytase to monogastric animal feed. The commercially available phytase is produced by *Aspergillus ficuum* and *Aspergillus niger* strains by submerged fermentation (SF). The cost of commercial phytase supplementation, however, is about $ 2−3/metric ton feed (*16*, *17*). A more economical alternative for enzyme production and application would be solid-state fermentation (SSF) (*2*). Many enzymes and other biochemicals can be produced by SSF at a fraction of the cost of SF production (*7*). 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, fungal protein and organic acids that increase feed digestibility and access to phytate in plant cells (*3*, *4*, *11*).

Various filamentous fungi such as *Mucor*, *Aspergillus*, *Rhizopus* spp. produce phytase and accessory enzymes by SSF (*11*). These fungi are substrate-specific, e.g., *A. ficuum* NRRL 3135 produces phytase best on wheat bran

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(*3*, Bogar et al., submitted), *Mucor racemosus* NRRL 1994 on coconut oil cake (present paper), and *Rhizopus oligosporus* NRRL 5905 on canola meal or on coconut oil cake (*10*). The availability and need for different feed ingredients thus influences the choice of fungi for phytase production.

In this paper phytase production by three *Mucor* and eight *Rhizopus* strains was compared in SSF on three feed ingredients commonly used in animal feed rations. These fungi, especially *M. racemosus* and *R. oligosporus* are widely used in SSF for production of fermented foods such as sufu and tempeh. The substrate composition was optimized by an 11-factor Plackett−Burman and a central composite design for *M. racemosus* NRRL 1994. Phytase and accessory enzyme production by *M. racemosus* NRRL 1994 was compared in solid state and shake flask fermentation, using coconut oil cake under optimized conditions.

Materials and Methods

Microorganisms and Inoculum Preparation. *Mucor hiemalis* ATCC 26035, *M. hiemalis* NRRL 13009, *M. racemosus* NRRL 1994, *Rhizopus microsporus* NRRL 3671, *R. oligosporus* NRRL 5905, *R. oryzae* ATCC 34121, *R. oryzae* NRRL 1526, *R. oryzae* NRRL 1891, *R. oryzae* NRRL 3562, *R. oryzae* NRRL 6431, *R. thailandensis* NRRL 6400 (= *R. oryzae* ATCC 20344), and *A. ficuum* NRRL 3135 strains were obtained from the American Type Culture Collection (ATCC), Manassas, VA and from Northern Regional Research Center (NRRL), USDA, Peoria, IL. The fungi were grown and maintained on potato dextrose agar (PDA) Petri plates and slants. Viable spores from 6-day-old fully sporulated slants were harvested by washing with 0.1% Tween-80 containing water, and the spore suspension was adjusted to 10^6 CFU (colony forming units on PDA plates) per mL for inoculation.

Substrates. The substrates were obtained from the following suppliers: defatted canola meal (Archer Daniels Midland, Velva, ND); coconut oil cake (local mill, Trivandrum, India); untreated corn fiber (Williams Companies, Perkin, IL); sesame oil cake (local mill, Trivandrum, India); spent brewing grain (Dreher Brewery, Budapest, Hungary); wheat bran (local market, Budapest, Hungary).

Solid State Fermentation (SSF) in Erlenmeyer Flask. A 10-g air-dried sample substrate was placed in a 500-mL cotton-plugged Erlenmeyer flask, supplemented with 23 mL of salt solution containing (g/L): NH_4NO_3, 5; $MgSO_4 \cdot 7H_2O$, 1; NaCl, 1. During the screening of substrates at different moisture levels 10 g air-dried substrate 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 moisture (35%, 55%, 64%, 71%, 75%, 78%, 80%) was adjusted by adding water. The wet substrate was sterilized at 121 °C for 20 min. After cooling the substrate was inoculated with 1 mL spore suspension (10^6 CFU/mL) of the respective fungus. The flasks were incubated for 3 days at 25 ± 1 °C, unless otherwise noted. All experiments were carried out in duplicate. The results shown are average values; error bars on graphs represent data points.

Submerged Fermentation (SF) in Shake Flasks. A 5-g air-dried sample of coconut oil cake (ground in a coffee mill) was suspended in 100 mL of solution containing 0.23 g glucose, 0.19 g casein, 0.15 g $(NH_4)_2SO_4$, in a cotton-plugged Erlenmeyer flask, sterilized at 121 °C for 20 min and then inoculated with 1 mL of spore suspension of *M. racemosus* NRRL 1994 containing 10^6 CFU/mL (2×10^5 CFU/g on dry matter of substrate in the final medium). The flasks were incubated in a rotary shaker at 200 rpm, 25 ± 1 °C.

Analytical Techniques. Enzyme activities were determined from samples of culture broth (SF) or culture extract (SSF). The SSF samples were extracted with water containing 0.1% Tween-80 (10 mL water/g dry substrate), by shaking 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.

α-Amylase activity was determined as described by Okolo et al. (*18*). The reaction mixture consisted of 1.25 mL of 1% soluble starch (E. Merck, Darmstadt, Germany) solution, 0.25 mL of 0.1 M sodium acetate buffer (pH 5.0), 0.25 mL of distilled water, and 0.25 mL of properly diluted crude enzyme solution. After 10 min of incubation at 50 °C, the liberated reducing sugars (glucose equivalent) were estimated by the dinitrosalicylic acid method of Miller (*19*). The color developed by the liberated reducing sugars was measured spectrophotometrically at 540 nm. One unit of α-amylase is defined as the amount of enzyme releasing 1 μmol of glucose equivalent per minute under assay conditions.

Phytase activity was assayed by measuring the inorganic phosphorus released from sodium phytate solution using the method described by Harland and Harland (*14*). The reaction mixture consisted of 1 mL of 0.1 M $MgSO_4 \cdot 7H_2O$, 2.4 mL of 6.82 mM phytic acid, and 0.6 mL of properly 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 reaction was carried out at 55 °C for 60 min and was stopped by adding 0.5 mL of 10% trichloroacetic acid. Blue color was measured spectrophotometrically at 660 nm after adding 2.5 mL of Taussky−Schoor reagent (*12*). One unit of phytase is defined as the amount of enzyme releasing 1 μmol of inorganic phosphorus per minute under the assay conditions.

Effect of Temperature and pH on Phytase Activity. To determine the optimum temperature of phytase activity the assay was performed at 25, 35, 45, 50, 55, 65, and 70 °C at pH 5.15 for 60 min using extract of SSF sample. To study the optimum pH of enzyme activity the assay was performed at 55 °C for 60 min, in pH range of 3.0−8.0. Buffers used were 0.1 M citric acid/sodium citrate buffer (pH 3.0−3.5), 0.2 M acetic acid/sodium acetate buffer (pH 4.0−6.0), 0.1 M Tris/HCl (pH 7.0−8.0).

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) (*20*). 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 of *p*-nitrophenol per minute under the assay conditions.

Lipase activity was determined by a spectrophotometric assay with *p*-nitrophenyl laurate (pNPL) as substrate (*21*). Solution A contained pNPL (76.6 mg) dissolved in propane-2-ol (30 mL); solution B contained Triton X-100 (2 g) and gum arabic (0.5 g) dissolved in 450 mL of buffer (Tris-HCl 50 mM, pH 7.5). The assay solution was prepared by adding 1 mL of solution A to 9 mL of solution B dropwise to get an emulsion that remained stable for 2 h. The assay mixture contained 2

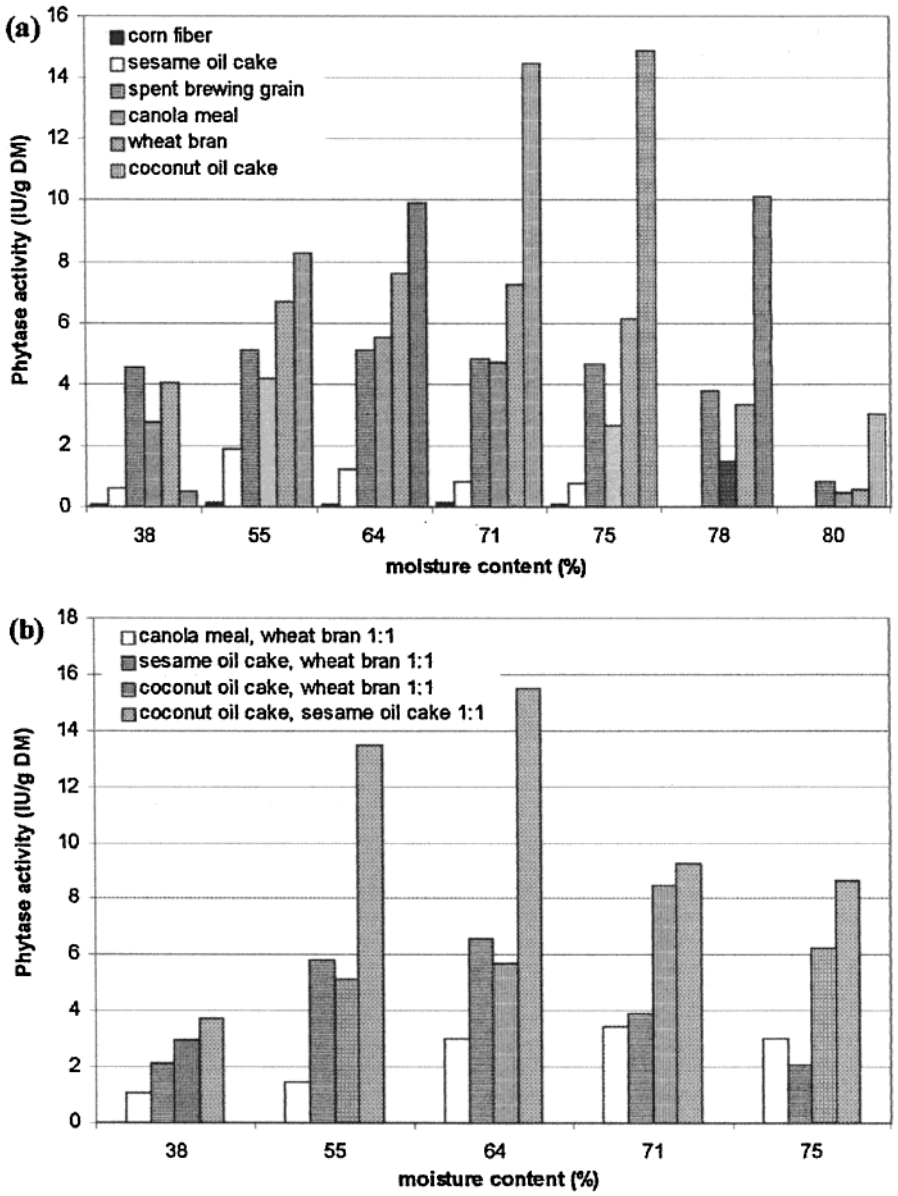

Figure 1. Effect of moisture content on phytase production of *Mucor racemosus* NRRL 1994 in solid-state fermentation on different agro-industrial residues: (a) SSF on monosubstrates; (b) SSF on mixed substrates. Fermentation conditions: 10 g of air-dried substrate in a 500-mL cotton-plugged Erlenmeyer flask was supplemented with 5 mL of salt solution containing (g/L) NH_4NO_3 23, $MgSO_4 \cdot 7H_2O$ 4.6, NaCl 4.6. Different moisture levels (38%, 55%, 64%, 71%, 75%, 78%, 80%) were adjusted by adding water. Inoculum: 10^5 spores/g substrate. Incubation temperature: 25 ± 1 °C. Fermentation time: 3 days.

mL of the emulsion and 0.5 mL of the appropriately diluted enzyme solution. The reaction was carried out at 45 °C for 30 min. The absorbance of liberated *p*-nitrophenol was measured at 410 nm. One unit of lipase activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol/min under test conditions.

Soluble protein was determined spectrophotometrically according to the method described by Lowry et al. (*22*).

Experimental Design. Plackett−Burman screening design allowed testing of multiple independent variables within a single experiment (*23*). After the independent variables and their corresponding levels had been selected, the experiments were performed and the responses, such as enzyme production, were measured. These experiments were carried out in flasks using 10 g of air-dried coconut oil cake plus 11 medium ingredients, including three carbon sources, three organic nitrogen sources, four inorganic nitrogen sources and one inorganic phosphate source, under conditions described above at constant 71% 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 was considered to be significant if $t_{\alpha/2} < t$ (*12*). $t_{\alpha/2} = 2.179$ at the probability level of $\alpha = 0.05$ and degrees of freedom was 12. The most common mean of assessing significance, the *p*-value, was also evaluated for each factor. The *p*-value is the probability that the magnitude of a parameter estimate is due to random process variability (*24*).

Central composite design was carried out in order to find the optimal concentrations of the three variables previously selected by the Plackett−Burman design (*25*, *26*). Using this statistical method, each factor can be set at five levels, coded $-\alpha$, -1, 0, $+1$, $+\alpha$. For optimizing three factors, the experimental design includes a full 2^3 factorial design. Nine possible combinations of the two factors at level -1 or $+1$ were augmented with two

Table 1. Best Phytase Producers in Screening of Three *Mucor* and Eight *Rhizopus* Strains; *Aspergillus ficuum* NRRL 3135 Was Used as Control Strain[a]

	phytase activity (IU/g DM)		
	canola meal	coconut oil cake	wheat bran
Mucor racemosus NRRL 1994	4.7	14.5	7.3
Mucor hiemalis NRRL 13009	0.1	11.5	0.8
Rhizopus microsporus NRRL 3671	1.3	0.4	0.5
Rhizopus oligosporus NRRL 5905	5.4	2.6	0.4
Rhizopus oryzae NRRL 1891	4.3	5.3	0.4
Rhizopus oryzae NRRL 3562	3.0	5.5	0.4
Rhizopus thailandensis NRRL 6400	1.5	2.7	0
Aspergillus ficuum NRRL 3135	5.2	7.8	11.8

[a] Fermentation conditions: 10 g of air-dried substrate in 500-mL cotton-plugged Erlenmeyer flask was supplemented with 23 mL of salt solution containing (g/L) NH_4NO_3 5, $MgSO_4 \cdot 7H_2O$ 1, NaCl 1. Moisture level: 71%. Inoculum: 1 mL of spore suspension of the respective fungus. Incubation temperature: 25 ± 1 °C. Fermentation time: 3 days.

Table 2. Assigned Concentrations of Coconut Oil Cake Supplements at Different Levels of the Plackett−Burman Design for 11 Factors

		levels (g/g dry substrate %)		
factor	symbol	−1	0	+1
starch	A	0.46	2.53	4.6
glucose	B	0.46	2.53	4.6
sucrose	C	0.46	2.53	4.6
soybean meal	D	0	4.9	9.8
corn steep liquor	E	0	9.2	18.4
casein	F	0	2.42	4.83
NH_4NO_3	G	0	0.43	0.85
NH_4Cl	H	0	0.58	1.15
$(NH_4)_2SO_4$	I	0	0.72	1.43
KNO_3	J	0	1.08	2.16
KH_2PO_4	K	0	0.58	1.15

replications of the center point and the six star points. Star points had one factor an axial distance to the center of ±α, whereas the other factor was at level 0. The axial distance α was chosen to be 1.764 to make this design orthogonal.

Statistica for Windows (StatSoft Inc.) was used for the regression analysis of the experimental data obtained. For determination of 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

Preliminary Experiments. *Comparison of Fungi for Phytase Production.* In a preliminary experiment, three *Mucor* and eight *Rhizopus* strains were screened for phytase production on canola meal, coconut oil cake and wheat bran in 3-day SSF. The results are shown in Table 1. *Aspergillus ficuum* NRRL 3135, which is frequently cited in the literature was used as control strain. The best producer was *M. racemosus* NRRL 1994 (= ATCC 46129) on coconut oil cake. This particular isolate is a safe microorganism since it is used in sufu fermentation (*27*).

Effect of Substrate and Moisture on Phytase Production. The effect of moisture content on phytase production on different substrates was established (Figure 1a and b). The best production was achieved on coconut oil cake at 71−75% moisture content (14.5 IU/g DM) and on a 1:1 mixture of coconut and sesame oil cake at 64% moisture content (15.5 IU/g DM) by *M. racemosus*

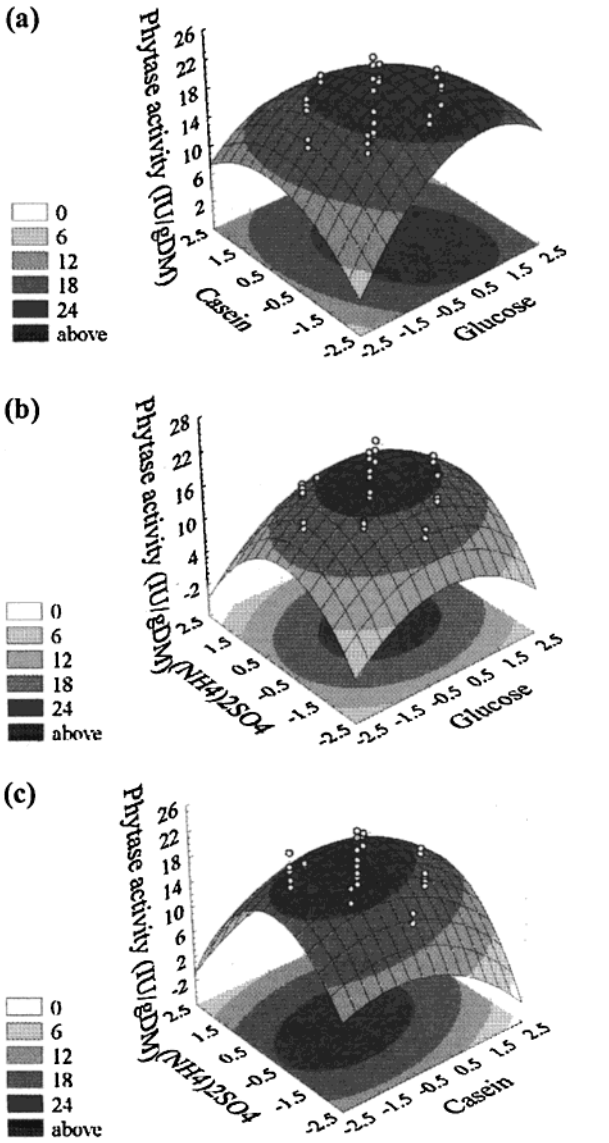

Figure 2. Surface plots reflecting the effect of glucose, casein and ammonium sulfate on phytase production of *Mucor racemosus* NRRL 1994 in SSF.

NRRL 1994. On the basis of these preliminary studies *M. racemosus* NRRL 1994 was selected for a systematic study of optimization using coconut oil cake and various nutritional supplements in solid-state fermentation.

Optimization of Nutrient Composition for Phytase Production. Coconut oil cake was supplemented with 11 ingredients, which most likely influence enzyme production by *M. racemosus* NRRL 1994. These include carbohydrates, nitrogen sources and phosphate as shown in Table 2. The effect of these 11 ingredients was studied in an 11 factors Plackett−Burman experimental design (Table 3). The regression coefficients and *t* and *p* values for the 11 ingredients are presented in Table 4. The coefficient of determination R^2 was calculated to be 0.98. This indicates that the model explains 98% of the variability in the data. Glucose, casein, corn steep liquor and ammonium salts constituted positive effects on the production of phytase by the fungal culture. For further optimization by central composite design glucose was chosen as carbon source, casein as organic nitrogen source and ammonium sulfate as inorganic nitrogen source (Table 5). The design of this experiment and the experimental results are shown in Table 6. Analysis of variance was performed in order to validate the regres-

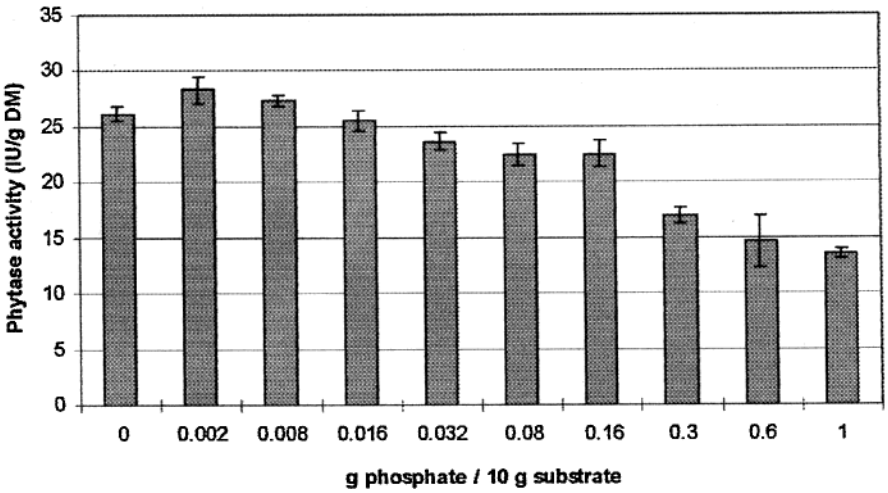

Figure 3. Effect of phosphate concentration on phytase production of *Mucor racemosus* NRRL 1994. Fermentation conditions: 10 g of air-dried coconut oil cake in a 500-mL cotton-plugged Erlenmeyer flask was supplemented with 23 mL of nutrient solution containing (g/L) glucose 19.6, casein 16.7, $(NH_4)_2SO_4$ 13.0, and different amounts of KH_2PO_4. Inoculum: 10^5 spores/g substrate. Incubation temperature: 25 ± 1 °C. Fermentation time: 3 days.

Table 3. Plackett−Burman Design for 11 Factors and Experimental Results[a]

trial	factors											phytase activity
no.	A	B	C	D	E	F	G	H	I	J	K	(IU/g DM)
1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	15.0
2	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	22.1
3	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	12.3
4	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	1.2
5	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	6.4
6	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	22.7
7	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	23.0
8	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	16.9
9	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	19.1
10	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	22.1
11	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	13.9
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	8.6
13	0	0	0	0	0	0	0	0	0	0	0	14.8

[a] Fermentation conditions: 10 g of air-dried coconut oil cake and different additives (see Table 1). Moisture level: 71%. Inoculum: 10^5 spores of *M. racemosus* NRRL 1994/g substrate. Incubation temperature: 25 ± 1 °C. Fermentation time: 3 days.

Table 4. Results of Regression Analysis for the Plackett−Burman Design[a]

term	coefficient	*t* value	*p* value
intercept	15.23	55.94	0.0000
A	−0.36	−1.28	0.2224
B	1.46	5.16	0.0001
C	−0.08	−0.27	0.7920
D	−0.49	−1.73	0.1075
E	1.32	4.64	0.0005
F	1.71	6.07	0.0000
G	0.95	3.35	0.0052
H	3.01	10.63	0.0000
I	3.67	12.95	0.0000
J	−0.52	−1.83	0.8980
K	−3.98	−14.04	0.0000

[a] Coefficient of determination $R^2 = 0.98$.

sion model. The regression coefficients and *t* and *p* values are presented in Table 7. The fit of the model is expressed by R^2, which was calculated to be 0.84. The model therefore accounts for 84% of the response. The three-dimensional graphs obtained from the calculated response surface are presented in Figure 2a−c. The coordinates of the maximum point were found to be $X_1 = 0.33$, $X_2 = -0.63$ and $X_3 = -0.17$ corresponding to the optimal supplementation levels for glucose, casein and ammonium sulfate of 4.51, 3.84 and 2.99 g/100 g dry coconut oil cake, respectively. In all further experiments coconut oil cake was supplemented with these optimized levels of glucose, casein and ammonium sulfate. It appeared from the optimization study that the optimal substrate composition, especially the quality of the nitrogen source is critical for maximizing phytase production. A similar trend was observed in optimization of the nutrient composition of a wheat bran medium for *A. ficuum* NRRL 3135 (Bogar et al., submitted for publication).

Table 5. Concentrations of Variables Assigned to the Different Levels of the Central Composite Design

		levels (% g/g dry substrate)				
factor	symbol	−α	−1	0	+1	+α
glucose	X_1	0.64	2.07	3.91	5.75	7.18
casein	X_2	1.22	3.0	5.3	7.6	9.36
$(NH_4)_2SO_4$	X_3	0.78	1.84	3.22	4.6	5.66

Table 6. Central Composite Design with the Coded Values X_i of the Independent Variables and Experimental Design[a]

trial	coded levels			phytase activity
no.	X_1	X_2	X_3	(IU/g DM)
1	−1	−1	−1	17.7
2	−1	−1	+1	15.9
3	−1	+1	−1	16.8
4	−1	+1	+1	16.4
5	+1	−1	−1	20.0
6	+1	−1	+1	18.3
7	+1	+1	−1	15.6
8	+1	+1	+1	16.7
9	0	0	0	19.6
10	−α	0	0	15.6
11	+α	0	0	17.3
12	0	−α	0	19.5
13	0	+α	0	15.9
14	0	0	−α	15.1
15	0	0	+α	12.8
16	0	0	0	22.8

[a] Fermentation conditions: 10 g of air-dried coconut oil cake and different additives (see Table 4). Moisture level: 71%. Inoculum: 10^5 spores of *M. racemosus* NRRL 1994/g substrate. Incubation temperature: 25 ± 1 °C. Fermentation time: 3 days.

Effect of Phosphate on Phytase Production. Since phosphate may be involved in the regulation of the phytase productivity (*28*) the influence of phosphate addition to the optimized medium was investigated. This

Table 7. Results of Regression Analysis of the Central Composite Design[a]

term	coefficient	*t* value	*p* value
intercept	21.21	36.17	0.0000
X_1	0.48	2.19	0.0396
X_2	−0.90	−4.09	0.0005
X_3	−0.47	−2.15	0.0432
X_1X_2	−0.72	−2.44	0.0234
X_1X_3	0.19	0.66	0.5176
X_2X_3	0.52	1.79	0.0885
X_1^2	−1.37	−5.34	0.0000
X_2^2	−9.63	−3.75	0.0012
X_3^2	−2.17	−8.45	0.0000

[a] Coefficient of determination $R^2 = 0.84$.

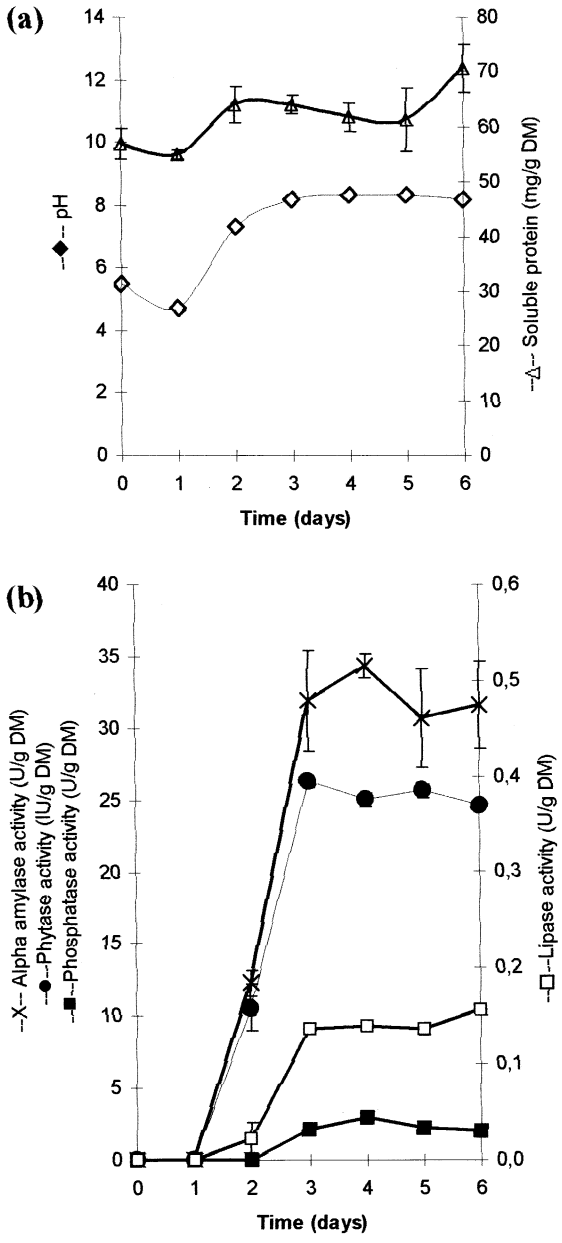

Figure 4. Kinetics of solid-state fermentation by *Mucor racemosus* NRRL 1994 in a 500-mL Erlenmeyer flask on optimized medium. Fermentation conditions: 10 g of air-dried coconut oil cake was supplemented with 23 mL of nutrient solution containing (g/L) glucose 19.6, casein 16.7, $(NH_4)_2SO_4$ 13.0. Inoculum: 10^5 spores/g substrate. Incubation temperature: 25 ± 1 °C.

phosphate addition represented a wider range of concentration than applied in Plackett−Burman experimental

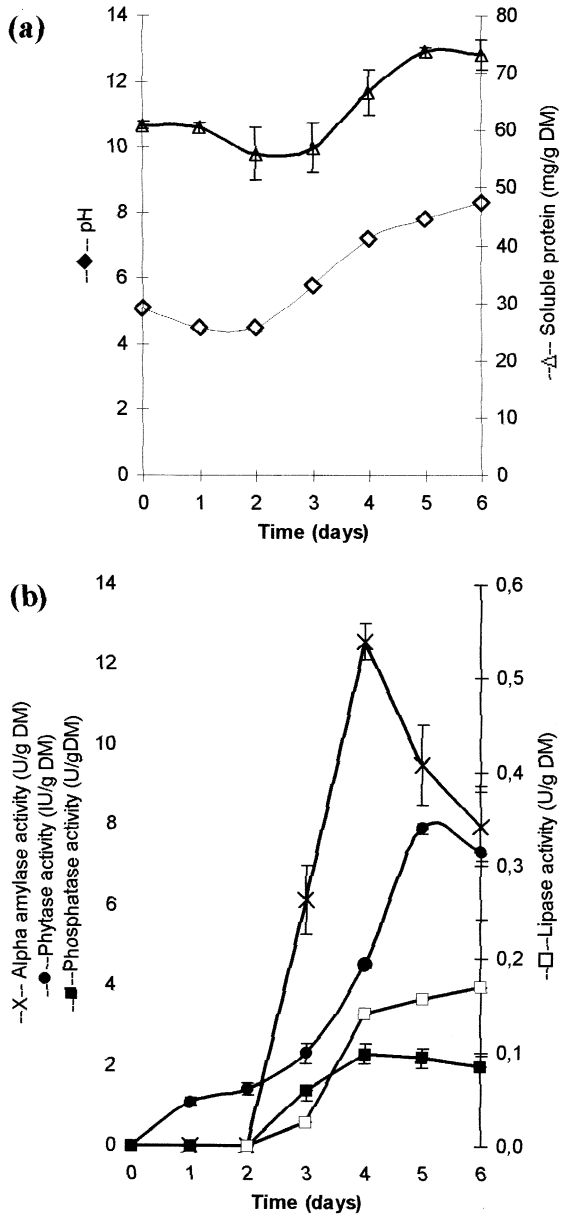

Figure 5. Kinetics of submerged fermentation by *Mucor racemosus* NRRL 1994 in Erlenmeyer flask. Fermentation conditions: 100 mL of medium (pH 5.1) in 500-mL Erlenmeyer flasks, containing 5 g of coconut oil cake powder, 0.23 g of glucose, 0.19 g of casein, 0.15 g of $(NH_4)_2SO_4$. Inoculum: 10^4 spores/mL medium. The flasks were incubated in rotary shaker at 200 rpm and 25 ± 1 °C. The activities are calculated on dry material (DM) of the initial fermentation medium.

design. Air-dried coconut oil cake (10 g) was supplemented with optimized additives and phosphate (KH_2PO_4) (0−1 g/10 g substrate) and then placed in 500-mL cotton-plugged Erlenmeyer flasks. The results are shown in Figure 3. A small amount of phosphate stimulated phytase production most likely by enhancing fungal growth, but levels greater than 0.1% inhibited phytase production.

Kinetics of Phytase Production by *Mucor racemosus* NRRL 1994. Phytase production was compared on optimized coconut oil cake medium in solid-state fermentation in stationary flasks (Figure 4) and in shake flask fermentation (SF) (Figure 5). The pH and soluble (secreted) protein levels were similar in SF and SSF. The maximal phytase production was 26 IU/g DM in 3 days in solid-state fermentation, but only 7.6 IU/g DM in 6 days in shake flask fermentation. This is comparable to

phytase production by *A. ficuum* NRRL 3135 on an optimized wheat bran medium: 25 IU/g DM in 4 days in solid-state fermentation, and 3.5 IU/g DM in shake flask fermentation (Bogar et al., submitted for publication). The production of accessory enzymes by the two strains, however, were different. *Aspergillus ficuum* NRRL 3135 produced 42 U/g DM phosphatase in SSF and 22 U/g DM in SF, whereas *M. racemosus* NRRL 1994 produced only 3.0 U/g DM phosphatase in SSF and 2.3 U/g DM in SF. α-Amylase production of *M. racemosus* NRRL 1994 was similarly less (35 U/g DM in SSF and 13 U/g DM in SF) compared to that of *A. ficuum* NRRL 3135 (130 U/g DM in SSF and 22 U/g DM in SF). Since coconut oil cake contains 8% residual oil, lipase activity was determined. *Mucor racemosus* NRRL 1994 produced 0.16 U/g DM lipase both in SSF and SF. These results indicate that selecting a fungus for phytase production, substrate availability, fungal preference for a given substrate and potential accessory enzyme production also should be considered. The results also indicate clearly that the crude SSF enzyme is a better source for both phytase and most accessory enzyme production than the SF product. The SSF enzyme is easily mixed with other ingredients in a feed ration in contrast to the SF product that is highly diluted. The economy of the SSF enzyme appears to be favorable. Assuming a modest phytase production of 25 IU/g DM substrate, a recommended 400 IU/kg feed supplementation would require only 16 kg crude SSF enzyme (whole SSF culture) to be added to each metric ton (MT) of feed. By using an assumed fermentation cost of $50/MT of substrate, the estimated cost could be an affordable $ 0.8/MT feed. In the literature phytase production by SF is in a range of 0.1−15 IU/mL (*3*, *28*, *29*) 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 SSF enzyme. To have the same economy as SSF, about 100 IU/mL SF production would be necessary to achieve the same $ 0.8/MT enzyme cost. (Assumption: 5 L crude enzyme/MT feed at 400 IU/kg feed level. At $200/m^3 fermentation cost the 5 L enzyme would cost $0.8). 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, and thus the potential for genetic improvement is very good.

Effect of Temperature and pH on Phytase Activity. The temperature and pH optimum of phytase produced by *M. racemosus* NRRL 1994 in solid-state fermentation was 55 °C (incubation time, 60 min) and 5.5, respectively (Figures 6 and 7). These values are very similar to other fungal phytases published by different authors. Most isolated phytases are active within the pH range 4.5−6.0 and show high activity in the temperature range of 50−70 °C (*11*, *30*). Two distinct pH optima were identified in the phytases of *Aspergillus* sp. 5990 and *A. ficuum* NRRL 3135; highest activity was displayed at pH 5.0−6.0 and a small second peak occurred at pH 2.5−2.8 (*28*). Maximum activity of phytase from *A. ficuum* NRRL 3135 was at 55 °C, from *Aspergillus* sp. 5990 at 60−65 °C (*28*) and from *Aspergillus niger* ATCC 9142 at 65 °C (*30*).

Conclusions

Phytase may be produced efficiently by *M. racemosus* NRRL 1994 in solid-state fermentation on optimized coconut oil cake at 71% moisture level, pH 5.5, incubation temperature 25 °C. By optimized nutrient supplementation and selecting the most appropriate carbon and nitrogen sources production nearly doubled (from 14.5 IU/g DM to 26 IU/g DM). The quality of carbon and nitrogen sources appears to be critical factors for maximal phytase production. The SSF-produced enzyme complex is an enriched feed ingredient that contains phytase in much higher concentrations than in the crude SF product. The SSF enzyme is easily mixed with other ingredients in a feed ration in contrast to the SF product that is highly diluted. Solid-state fermentation appears to be a more efficient, less costly, more directly applicable process for phytase supplementation to animal feed rations than the currently available submerged fermentation technology. The presence of accessory enzymes in the SSF product greatly enhances its value, by increasing feed digestibility and access of phytate to phytase attack. The SSF enzyme would compete favorably with the best available commercial phytase preparations.

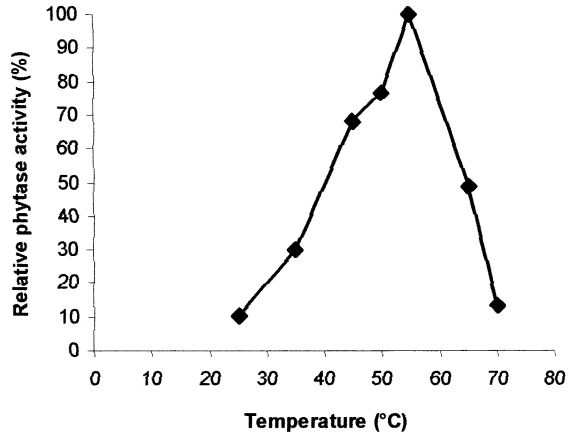

Figure 6. Effect of temperature on hydrolysis of phytic acid by phytase of *Mucor racemosus* NRRL 1994. Reaction was performed at pH 5.15 for 60 min.

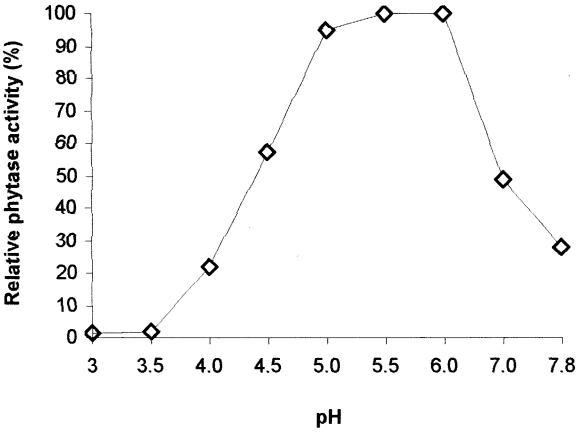

Figure 7. Effect of pH on hydrolysis of phytic acid by phytase of *Mucor racemosus* NRRL 1994. Reaction was performed at 55 °C for 60 min. Buffers used were 0.1 M citric acid/sodium citrate buffer (pH 3.0−3.5), 0.2 M acetic acid/sodium acetate buffer (pH 4.0−6.0), and 0.1 M Tris/HCl (pH 7.0−8.0).

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