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ORIGINAL PAPER

High level phytase production by *Aspergillus niger* NCIM 563 in solid state culture: response surface optimization, up-scaling, and its partial characterization

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Abstract Phytase production by *Aspergillus niger* NCIM 563 was optimized by using wheat bran in solid state fermentation (SSF). An integrated statistical optimization approach involving the combination of Placket–Burman design (PBD) and Box–Behnken design (BBD) was employed. PBD was used to evaluate the effect of 11 variables related to phytase production, and five statistically significant variables, namely, glucose, dextrin, $NaNO_3$, distilled water, and $MgSO_4{\cdot}7H_2O$, were selected for further optimization studies. The levels of five variables for maximum phytase production were determined by a BBD. Phytase production improved from 50 IU/g dry moldy bran (DMB) to 154 IU/g DMB indicating 3.08-fold increase after optimization. A simultaneous reduction in fermentation time from 7 to 4 days shows a high productivity of 38,500 IU/kg/day. Scaling up the process in trays gave reproducible phytase production overcoming industrial constraints of practicability and economics. The culture extract also had 133.2, 41.58, and 310.34 IU/g DMB of xylanase, cellulase, and amylase activities, respectively. The partially purified phytase was optimally active at 55°C and pH 6.0. The enzyme retained ca. 75% activity over a wide pH range 2.0–9.5. It also released more inorganic phosphorus from soybean meal in a broad pH range from 2.5 to 6.5 under emulated gastric conditions. Molecular weight of phytase on Sephacryl S-200 was approximately 87 kDa. The K_m and V_{max} observed were 0.156 mM and 220 μm/min/mg. The SSF phytase from *A. niger* NCIM 563 offers an economical production capability and its wide pH stability shows its suitability for use in poultry feed.

Keywords Phytase · *Aspergillus niger* · Solid state fermentation · Statistical methods · Response surface optimization

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Introduction

Phosphorus is an essential constituent of life like nitrogen, but, unlike nitrogen, phosphorus does not have a cycle to constantly replenish its supply. All animal diets must contain adequate amounts of this element. So to meet their phosphorus requirements, inorganic phosphorus especially dicalcium phosphate is supplemented in the diet of livestock and poultry animals. This has made phosphorus the third most expensive nutrient in poultry production after energy and protein. At the current extraction and usage rate, the existing phosphate reserves will be exhausted in next 80 years [35].

Bound phosphorus (18–88% of total phosphorus content) in fact exists as phytate which is already present in animal feed. But this phytate phosphorus is not utilized by monogastric animals like poultry and pigs due to lack of intrinsic phytase in their gastrointestinal tracts. Phytate in addition acts as an antinutrient by chelating various cations such as Ca^{2+}, Fe^{2+}, Zn^{2+}, and Mg^{2+} and thereby reducing their bioavailability. This unutilized phytate is the origin of phosphorus pollution as it builds up in areas of livestock production leading to eutrophication and algal blooms [22].

So use of phytase in animal feed will stop the antinutritional effects of phytate, decrease environmental

pollution, increase availability of starch, protein, amino acids, calcium, and phosphorus, and abolish the need for addition of inorganic phosphate in animal feed. Phytases are also imminent candidates for production of special isomers of different lower phosphate esters of *myo*-inositol, some of which are considered to be pharmacoactive and important intracellular secondary messengers [33].

The US Food and Drug Administration (FDA) has approved a "generally recognized as safe (GRAS)" petition for use of phytase in food, and phytase has been marketed as an animal feed enzyme in the US since 1996 [17]. All these factors have concurrently made phytase the third largest feed enzyme in terms of annual consumption [28].

There are various reports on phytase production by bacteria, yeast, and fungi among which fungal phytases are widely employed in animal feed due to their acid tolerance and higher yield [34]. The existing commercial microbial phytases produced by submerged fermentation (Smf) conditions are expensive because of diluted product, production using recombinant strains, and high product recovery costs [15]. SSF provides a more economic alternative for enzyme production and application as compared with Smf.

There is no defined medium for optimum production of phytase from different microbial sources especially fungi because each fungus has its own special conditions and specific substrates for maximum enzyme production especially in SSF. Krishna and Nokes studied the effect of culture conditions, particularly inoculum age, media composition (wheat bran and full-fat soybean flour), and duration of SSF, on the phytase production by *A. niger* [15]. Bogar et al. reported phytase production by *A. ficcium* NRRL 3135, *M. racemosus* NRRL 1994, and *R. oligosporous* NRRL 5905 using various substrates such as canola meal, cracked corn, soybean meal, and wheat bran [2]. But such reports are nevertheless few because of the low productivities and difficulties associated with operating and up-scaling SSF conditions [3].

There is need for intensive research on SSF to develop a commercial process for phytase production with techno-economic feasibility. The intricacies in SSF technology can be understood through modeling, kinetics of growth of microbes, control of parameters, optimization, scale-up and commercialization of the process for application. Before carrying out these studies it is, however, necessary to increase the phytase production and this can become significant by employing statistical optimization techniques rather than the conventional one variable at a time approach. This is because statistical optimization gives the optimum media formulation with minimum number of experiments in a short time while also considering the interaction between selected components [30].

Earlier, we reported phytase production by *A. niger* NCIM 563 under SSF using wheat bran with no additional nutrients [19]. The same fungus produces two dissimilar phytases under Smf [31]. In this paper, our objective is to evaluate the application of statistical methods to increase the phytase activity under SSF, partial characterization, and up-scaling to tray fermenters.

Materials and methods

Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals used were of analytical grade. Various agriculture residues were purchased from a local market.

Fungi and inoculum preparation

Aspergillus niger NCIM 563 used in the present study was from NCIM Resource Center, Pune, India. The stock cultures were maintained on potato dextrose agar (PDA) slants and stored at 4°C. Spores from 7-day-old PDA slants were harvested by using sterile distilled water containing 0.01% Tween 80 to obtain 5×10^7 spores/ml and used as inoculum for SSF.

SSF in Erlenmeyer flasks

The unoptimized medium contained 10 g of agricultural residue moistened with 10 ml distilled water in a 250-ml Erlenmeyer flask sterilized at 121°C for 30 min (Table 1). On cooling fermentation medium was inoculated with 1% spore suspension and incubated for 7 days at 30°C. The optimized fermentation medium for SSF contained 10 g of wheat bran in a 250-ml Erlenmeyer flask plus glucose 3 g, dextrin 1.25 g, sodium nitrate 0.2 g, magnesium sulfate 0.3 g moistened with 20 ml distilled water and sterilized by autoclaving at 121°C for 30 min. On cooling fermentation medium was inoculated with 1% spore suspension and incubated for 4 days at 30°C. Enzyme production was expressed as enzyme activity IU/g DMB. All experiments were carried out in triplicates.

SSF in trays

Wheat bran (50, 100, 200, and 1,000 g) was moistened with the optimized medium mentioned in "SSF in Erlenmeyer flasks" in enamel-coated metallic trays having dimensions $28 \times 24 \times 4$ cm, $45 \times 30 \times 4$ cm, and $80 \times 40 \times 4$ cm. The trays were covered with aluminum foil and sterilized at 121°C for 20 min. The cooled substrate was inoculated with 1% spore suspension of *A. niger* and incubated for 5 days at 30°C. Samples were withdrawn

Table 1 Properties of crude and partially purified phytase from *A. niger* NCIM 563

Enzyme	Property	Value
Crude	Production using agriculture residues	(IU/g DMB)
	Ground nut cake	36.21
	Coconut cake	18.28
	Cotton cake	38.56
	Wheat bran	50.0
	Rice bran	6.8
	Production in statistically optimized media	
	Phytase	154.8
	Xylanase	133.2
	Cellulase	41.58
	Amylase	310.34
	Up-scaling using optimized media	
	10 g WB in 250 ml flask	154
	50 g WB in 28 × 24 × 4 cm tray	150
	100 g WB in 28 × 24 × 4 cm tray	149
	200 g WB in 45 × 30 × 4 cm tray	151
	1,000 g WB in 80 × 40 × 4 cm tray	148
Partially purified	pH	
	Optimum	6.0
	Stability	2.0–9.5
	Temperature	
	Optimum	55°C
	Stability	20% residual activity at 60°C after 1 h
	Molecular mass	
	Gel filtration	87 kDa
	Effect of metal ions	
	Stimulated (5 mM)	Ca^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+}, Pb^{2}
	Inhibited (1 mM)	Hg^{2+}, Ni^{2+}, Zn^{2+}, $Cu^{2+,}$ Ag^{2+}
	K_m	0.156 mM
	V_{max}	220 μm/min/mg

aseptically each day from different parts of the tray to obtain representative composite samples.

Analytical methods

Phytase activity was measured at 50°C as described earlier [19]. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 μmol phosphorus per min under standard assay conditions.

Filter paper cellulase and xylanase activities were determined as reported earlier [11]. α-Amylase activity was determined by McCleary and Sheehan's method [21]. The reducing sugars were determined by using dinitrosalicylic acid (DNS) [20]. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of xylose, glucose, or galacturonic acid/min under the assay conditions. Protein concentration in the culture filtrate was determined by the method of Lowry et al. using bovine serum albumin as a standard [18]. Biomass determination was carried out by measuring the glucosamine content resulting from acid hydrolysis of the fermented substrate [32]. Glucosamine was determined by the method of Reissig and the dry mycelial weight was calculated by assuming a mycelia glucosamine content of 139 mg of glucosamine/g of dry mycelium [29].

Each experiment was carried out in triplicate and the values reported are the mean of three such experiments.

Partial purification of phytase

The enzyme extraction from Koji was done as mentioned by Mandviwala [19]. The filtrate obtained was subjected to ammonium sulfate precipitation (95% saturation) with constant stirring. The precipitate was collected by centrifugation (15,000×*g*, 20 min) and dissolved in minimum volume of acetate buffer (100 mM, pH 6.0). The enzyme was desalted by passing it through a Sephadex G-25 column and fractions were estimated for phytase activity.

Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) (8%) was performed at room temperature and 200 V for 2–3 h [6]. Protein bands were visualized by silver staining [7].

Molecular weight determination by gel filtration

The molecular weight of native enzyme was estimated by gel filtration on a Sephacryl S-200 (1 × 100 cm) column equilibrated with 20 mM acetate buffer, pH 5.5 using cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 KDa), alcohol dehydrogenase (150 kDa), and β-amylase(200 kDa) as standard proteins by the method of Andrews [1].

Characterization of phytase

The enzyme precipitated by ammonium sulfate and desalted by Sephadex G-25 was used for characterization of partially purified phytase. The optimum pH was determined by measuring the activity between pH 2.0 and 10.0 using 200 mM buffers: glycine–HCl (pH 2.0–3.0), sodium acetate (pH 4.0–6.0), Tris–HCl (pH 7.0–8.0), and glycine–NaOH (pH 9.0–10.0) at 50°C. Stability assay was performed by incubating the partially purified enzyme at 30°C for 24 h in 50 mM buffers of different pH values: glycine–HCl (pH 2.0–3.0), sodium acetate (pH 4.0–6.0), Tris–HCl (pH 7.0–8.0), and glycine–NaOH (pH 9.0–10.0). The residual activity was then assayed under standard assay conditions considering enzyme activity at zero time as 100%. The optimum temperature was determined over the temperature range 45–60°C. The thermal stability was studied up to 60°C and the residual enzyme activity was determined by using standard assay conditions and compared with the control without incubation.

To check the effect of metal ions, the enzyme was incubated in the presence of 1 mM and 5 mM of various metals ions for 30 min at 50°C under standard assay conditions along with control which is phytase reaction without metal ion.

The kinetic constants V_{max} and K_m were determined with sodium phytate as substrate using a Lineweaver–Burk plot [16].

Hydrolysis of soybean meal phytate in simulated gastric fluid

One gram soybean meal was dissolved in 9 ml simulated gastric fluid (SGF) [0.25 M glycine–HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin] and pH was adjusted with HCl or NaOH to a final pH of 1.5, 2.0, 2.5, 3.5, 5.5, or 6.5. The solutions were incubated with agitation at 37°C for 30 min, and pH was adjusted to the corresponding values again. Then 1 ml partially purified enzyme was added to the solutions and incubated by agitation at 37°C for 60 min. The amount of released phosphorus was determined by a modified ammonium molybdate method as described in "Analytical methods".

Optimization using response surface methodology

The PBD was employed to identify the key ingredients and the conditions for the best yield of enzyme production [26]. Phytase production is influenced by media components, especially carbon and nitrogen sources, metal ions, and physical variables such as pH, temperature, inoculum density, and incubation time. The choice of variables was made on the basis of studies employing Smf [31]. In the present study PBD was used to screen various variables, viz., glucose (*A*), dextrin (*B*), $NaNO_3$ (*C*), distilled water (*D*), $MgSO_4{\cdot}7H_2O$ (*E*), $FeSO_4{\cdot}7H_2O$ (*F*), KCl (*G*), incubation period (*H*), inoculum level (*J*), Triton X (*K*), and $MnSO_4$ (*L*). The total number of trials to be carried out was $n + 1$ where n is the number of variables. Each independent variable was tested at two levels, a high (1) level and a low (2) level. Table 2 shows the variables and their levels used in the experimental design constructed by using Design-Expert software (DES) version 7.1.2 (Stat-Ease, Minneapolis, MN, USA). The variables with more percent contribution were considered to influence phytase activity. Percentage values were calculated by adding the total sum of squares and then taking each term's sum of squares and dividing by the total.

On the basis of analysis of PBD results, five variables, viz., glucose (*A*), dextrin (*B*), $NaNO_3$ (*C*), distilled water (*D*), and $MgSO_4.7H_2O$ (*E*), were chosen for further optimization by response surface methodology (RSM) using BBD. BBD is a good design for RSM studies because it permits estimation of the best fit parameters of the quadratic model, building of

Table 2 PBD for selected factors and their assigned levels for phytase production with *A. niger* NCIM 563 in SSF

Sr no	Variable with designate	Level 1	Level 2
1	*A* Glucose (w/w %)	10	50
2	*B* Dextrin (w/w %)	5	20
3	*C* $NaNO_3$ (w/w %)	2	8
4	*D* Distilled water (ml)	100	200
5	*E* $MgSO_4.7H_2O$ (w/w %)	1	5
6	*F* $FeSO_4.7H_2O$ (w/w %)	1	5
7	*G* KCl (w/w %)	1	5
8	*H* Incubation period (days)	4	8
9	*J* Inoculum level (spores/ml)	0.01	100
10	*K* Triton X (v/v %)	0.5	1.0
11	*L* $MnSO_4$ (w/w %)	1	5

Table 3 Selected factors and their assigned levels by BBD for phytase production with *A. niger* NCIM 563 in SSF

Sr no	Variable with designate	Level 1	Level 2	Level 3
1	*A* Glucose (w/w %)	10	30	50
2	*B* Dextrin (w/w %)	5	12.5	20
3	*C* $NaNO_3$ (w/w %)	2	5	8
4	*D* Distilled water (ml)	100	150	200
5	*E* $MgSO_4 \cdot 7H_2O$ (w/w %)	1	3	5

sequential designs, and detection of lack of fit of the model [4]. The number of experiments (N) required for the development of BBD is defined as $N = 2k\ (k - 1) + C_o$ (where k is number of variables and C_o is the number of central points). This was used to develop a mathematical correlation between five variables on production of phytase. Each variable was studied at three levels (1, 2, and 3). The values for each component at three levels studied in the BBD are shown in Table 3. The design matrix was constructed by using DES to generate the response surface plot and optimum values for media formulation. The optimum values of the variables and the behavior of the system were studied by using the quadratic equation model in DES. All experiments were carried out in triplicates and their mean values are presented.

Results and discussion

Phytase production using agricultural residues

Wheat bran gave the highest phytase production of 50 IU/g DMB on the 7th day of fermentation as compared with groundnut cake, coconut cake, cotton cake, and rice bran (Table 1). As wheat bran is a cheaper substrate, economical, and supported maximum phytase production, it was selected for optimization experiments.

Optimization of phytase production by RSM

The 11 chosen variables (Table 2) likely to be influencing phytase production were optimized by using PBD. The PBD design matrix for experimental design of the 11 selected variables is shown in Table 4 along with their responses on phytase production. Maximum phytase production of 110 IU/g DMB was observed in trial number 11. The variables showing high percent contribution were considered as significant variables, influencing phytase production, by fitting to a linear model assuming the absence of interactions. Thus it was found that phytase production was influenced by glucose (A), dextrin (B), $NaNO_3$ (C), distilled water (D), and $MgSO_4 \cdot 7H_2O$ (E) as seen from the analysis of half normal plot and the total sum of squares and percent contribution respectively (Table 5). The remaining variables were observed to have small percent contribution and they were therefore considered insignificant. Vohra and Satyanarayana [34] also identified magnesium sulfate as an important variable for phytase production by the thermophilic mold *Sporotrichum thermophile* by using PBD. Moisture content of the medium in SSF is very important for the growth of microorganisms, production of enzyme, and for enzyme activity [27]. It was found that distilled water (DW) contributed the most to phytase production as observed from the analysis of PBD results. The best fit linear regression model used for

Table 4 Experimental designs used in PBD studies for 11 selected independent factors and also experimental values of phytase production

Trial number	Factor level											
	A Glucose	*B* Dextrin	*C* $NaNO_3$	*D* DW	*E* $MgSO_4$	*F* $FeSO_4$	*G* KCl	*H* Incubation period	*J* Inoculum level	K Triton X	L $MnSO_4$	Phytase production (IU/g DMB)
1	1	1	2	1	2	2	1	2	2	2	1	6.93
2	2	2	2	1	1	1	2	1	2	2	1	3.73
3	2	2	1	2	2	2	1	1	1	2	1	36.44
4	2	1	2	2	1	2	2	2	1	1	1	57.68
5	1	1	1	1	1	1	1	1	1	1	1	33.41
6	2	1	1	1	2	1	2	2	1	2	2	22.54
7	2	2	1	1	1	2	1	2	1	1	2	8.53
8	2	1	2	2	2	1	1	1	2	1	2	21.61
9	1	2	2	1	2	2	2	1	1	1	2	11.07
10	1	2	1	2	2	1	2	2	2	1	1	31.74
11	1	1	1	2	1	2	2	1	2	2	2	110.40
12	1	2	2	2	2	1	1	2	1	2	2	53.49

The culture was grown under solid state fermentation conditions at 30°C as described in "Material and methods". The values given are the average of three independent experiments

Table 5 Analysis of PBD for percent contribution of each variable and its effect on phytase production by *A.niger* NCIM 563

Variable code	Variable	Sum of squares	% Contribution
A	Glucose	776.23	7.85
B	Dextrin	964.15	9.75
C	$NaNO_3$	653.70	6.61
D	DW	4224.32	42.72
E	$MgSO_4{\cdot}7H_2O$	1562.06	15.80
F	$FeSO_4{\cdot}7H_2O$	347.06	3.51
G	KCl	490.85	4.96
H	Incubation period	106.46	1.08
J	Inoculum level	83.66	0.85
K	Triton X	402.44	4.07
L	$MnSO_4$	277.38	2.81

modeling gave a model *F* value of 5.75. This implies that there is only 0.01% chance that this "model *F* value" could occur due to noise. The coefficient of determination was obtained as $R^2 = 0.873$ and shows that the model used for analyzing the data is significant.

The five medium components (Table 3), identified above as significant variables for phytase production by PBD, were further optimized by RSM using BBD. Table 6 represents the design matrix of the five significant variables in coded levels, and reports the experimentally obtained phytase activity. It may be seen that trial number 20 showed the highest phytase activity. The experimental results obtained for phytase production were fed into the DES and analysis was carried out using backward elimination regression. The calculated regression equation for the optimization of medium components showed the phytase activity (*Y*) as a function of these variables. By applying multiple regression analysis on the experimental data, the following quadratic model was found to explain phytase production:

$$\begin{aligned} Y =& 31.04 - 10.66A + 3.42B - 3.45C + 46.99D + 3.50E \\ &+ 20.54AC - 6.75AD + 15.10AE - 16.48BC \\ &+ 16.35CE - 11.15DE - 9.30B^2 + 9.10C^2 \\ &+ 32.56D^2 + 7.80E^2 \end{aligned} \quad (1)$$

where *Y* is the predicted response and *A*–*E* are variables.

The results were analyzed by using ANOVA as appropriate to the experimental design used. The quality of the model was checked by using various criteria. The coefficient of determination (R^2) is 0.9499 for phytase production, suggesting that 94.99% of the variability is explained in the model. The value of correlation coefficient (predicted R^2) for phytase production was 0.8685, which suggests a strong agreement between the experimental and

Table 6 BBD for optimization of selected variables and obtained experimental values of phytase production using *A. niger* NCIM 563 in SSF

Trial number	Factor level					Phytase production (IU/g DMB)
	A Glucose	*B* Dextrin	*C* $NaNO_3$	*D* DW	*E* $MgSO_4$	
1	2	3	2	2	3	43.22
2	2	2	2	2	2	34.23
3	2	2	2	2	2	33.62
4	2	2	2	1	1	5.01
5	2	2	3	3	2	125.39
6	1	2	1	2	2	90.83
7	3	2	1	2	2	9.08
8	3	2	2	2	3	44.02
9	3	2	2	3	2	90.86
10	2	2	1	1	2	22.81
11	3	2	3	2	2	32.47
12	2	2	3	2	1	22.90
13	3	2	2	1	2	39.12
14	2	2	3	1	2	12.30
15	3	1	2	2	2	6.06
16	2	2	2	2	2	32.72
17	2	1	1	2	2	14.33
18	2	2	1	2	3	34.68
19	1	2	2	2	1	59.98
20	2	2	1	3	2	139.53
21	3	2	2	2	1	4.85
22	2	2	2	3	3	108.85
23	2	1	2	2	3	36.28
24	2	2	2	1	3	32.01
25	2	1	2	3	2	77.5
26	2	3	1	2	2	42.2
27	2	3	2	3	2	105.20
28	1	2	2	1	2	7.36
29	1	2	2	2	3	38.68
30	2	2	2	3	1	126.46
31	1	1	2	2	2	18.70
32	2	2	3	2	3	65.63
33	3	3	2	2	2	6.62
34	2	1	2	1	2	5.84
35	2	3	2	1	2	23.61
36	2	3	3	2	2	13.42
37	2	1	2	2	1	35.21
38	1	2	2	3	2	126.10
39	2	2	2	2	2	31.22
40	1	2	3	2	2	32.01
41	2	2	1	2	1	57.36
42	2	1	3	2	2	51.49
43	2	3	2	2	1	35.75
44	2	2	2	2	2	31.21

Table 6 continued

Trial number	Factor level					Phytase production (IU/g DMB)
	A Glucose	*B* Dextrin	*C* $NaNO_3$	*D* DW	*E* $MgSO_4$	
45	1	3	2	2	2	30.14
46	2	2	2	2	2	33.45

The culture was grown under solid state fermentation conditions at 30°C as described in "Material and methods". The values given are the average of three independent experiments

predicted values of phytase production. The model F value of 37.94 and values of $P > F$ (<0.0001) indicated that the model terms are significant. For phytase production, A, D, AC, AD, AE, BC, CE, DE, B^2, C^2, D^2, and E^2 are significant model terms. The results of the ANOVA study are presented in Table 7.

The results show a strong agreement between the predicted and the experimental response. The optimum values of the tested variables are obtained as glucose 3 g, dextrin 1.25 g, $NaNO_3$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.3 g moistened with 20 ml distilled water per 10 g of wheat bran with the rest of the variables kept at mean value of the corresponding ranges in PBD. The enzyme production behavior was then studied under optimized fermentation conditions by monitoring in time for 6 days. An unoptimized medium showed a phytase production of 50 IU/g DMB on the 7th day, whereas optimization studies gave phytase production of 154 IU/g DMB on the 4th day. The rapid growth of fungus is corroborated by the corresponding increase in the mycelial weight (Fig. 1). Thus the phytase activity is increased by 3.08 times with a simultaneous reduction of fermentation from 7 to 4 days. The three-dimensional (3D) plots showing the optimal levels and nonlinear interactions among the variables for enzyme production are presented in Fig. 2a, b. The maximum predicted value for phytase production was 123.14 IU/g DMB, while the experimental response was 139.53 IU/g DMB (run number 20 in Table 6). It may also be noted that optimization using PBD and central composite design in the case of *A. ficcium* gave phytase activity of only 15 IU/g using wheat bran [2].

Table 7 ANOVA for response surface reduced quadratic model of BBD

Source	Sum of squares	Mean square	F value	P value Prob > F
Model	57,056.16	3,803.74	37.94	<0.0001
A	1,819.43	1,819.43	18.15	0.0002
B	187.26	187.26	1.87	0.1819
C	190.26	190.26	1.9	0.1785
D	35,328.37	35,328.37	352.39	<0.0001
E	195.54	195.54	1.95	0.1728
AC	1,687.96	1,687.96	16.84	0.0003
AD	1,122.38	1,122.38	11.2	0.0022
AE	911.44	911.44	9.09	0.0052
BC	1,086.75	1,086.75	10.84	0.0025
CE	1,069.64	1,069.64	10.67	0.0027
DE	497.44	497.44	4.96	0.0336
B^2	816.23	816.23	8.14	0.0078
C^2	780.18	780.18	7.78	0.0091
D^2	9,995.29	9,995.29	99.70	<0.0001
E^2	573.38	573.38	5.72	0.0233
Lack of fit	2,999.40	119.98	73.45	<0.0001
Pure error	8.17	1.63		
Residual	3,007.57	100.25		
Cor total	60,063.73			

$R^2 = 0.9499$

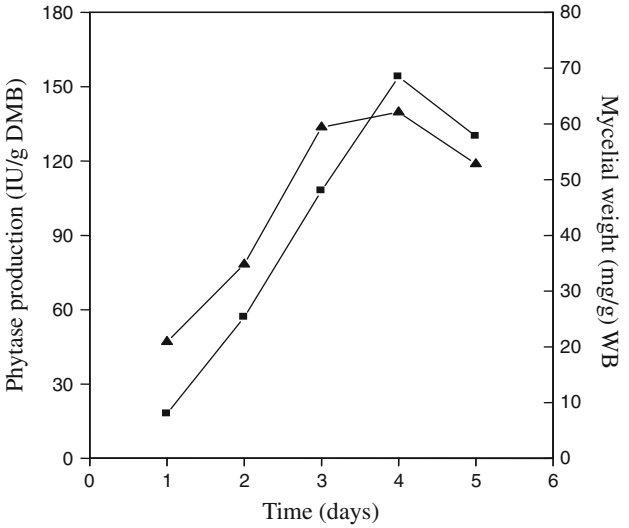

Fig. 1 Kinetics of phytase production in SSF by *A. niger* NCIM 563 under optimized conditions. Phytase production (IU/g DMB) (*filled squares*). Mycelial weight (mg/g WB) (*filled triangles*)

The optimized results show a productivity of 38,500 IU/kg/day which is the highest as compared with other reported SSF using spore inoculum. Our experiments for phytase production using vegetative inocula did not give significant increase in production (data not included) but our productivity of 38,500 IU/kg/day is 8.3-fold more as compared with 4,667 IU/kg/day reported for *A. niger* by Krishna and Nokes (Table 8). Moisture content in SSF plays a crucial role as can be seen from the above response surface analysis. But increasing the moisture content leading to semi-solid conditions did not enhance the activity any further. Thus the above optimized variables using response surface analysis gave the best conditions for maximum phytase production.

A recommended 400 IU/kg feed supplementation would require only 2.59 kg crude SSF enzyme of *A. niger* NCIM 563 to be added to each metric ton (MT) of feed

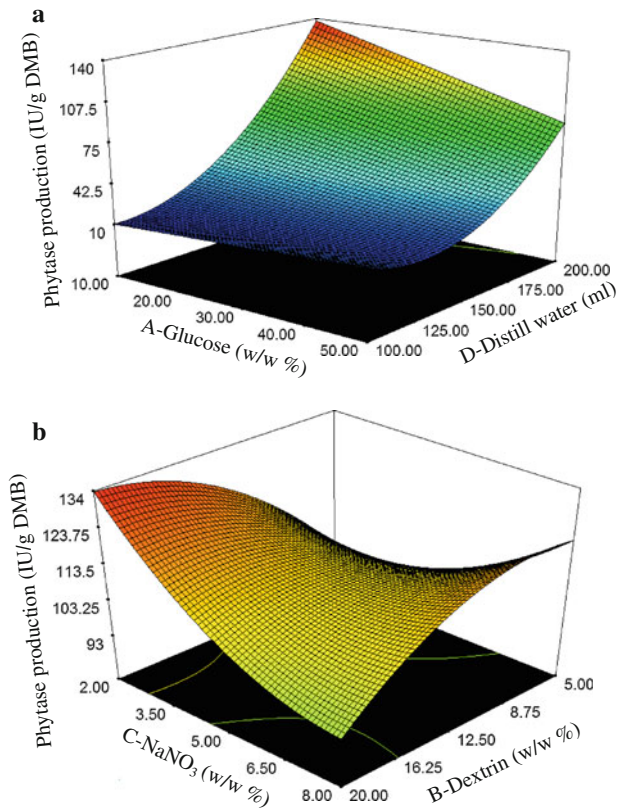

Fig. 2 3D surface plots reflecting the effect of glucose and distilled water (**a**) and dextrin and sodium nitrate (**b**) on phytase production by *A. niger* NCIM 563 in SSF

Table 8 Comparison of phytase production by *A. niger* NCIM 563 with other fungal strains grown under SSF

Phytase source	Phytase activity (IU/g DMB)	Phytase productivity (IU/kg/day)	Reference number
Rhizopus pusilis	9.18	76	[5]
Rhizopus thailiandenism	3	38	[34]
Rhizopus oligosporous	5	75	[34]
Rhizopus microsporous	1	18	[34]
Mucor racemosus	26	361	[3]
Mucor hiemalis	12	160	[3]
Aspergillus niger	1,008[a]	4,667[a]	[15]
Aspergillus ficcium	15	159	[2]
Aspergillus niger CFR 335	70	14,000	[12]
Aspergillus niger NCIM 563	154	38,500	Present work

[a] Production using vegetative inoculum

which presently requires 16 kg crude SSF enzyme of *A. ficcium* NRRL 3135. At present all available commercial phytase preparations are produced by recombinant strains using Smf. But they are costly as it requires concentration of diluted enzyme, extensive downstream procedures, and treatment of generated effluents that do not make the process eco-friendly. All this restricts the use of Smf phytase in animal feed [25]. In contrast, as compared with Smf, the SSF enzyme as shown here is produced in large quantity along with hydrolytic enzymes and its application involves minimum downstream processing because the product can now be easily mixed with other ingredients in a feed ration [24]. So the process economy of the SSF enzyme appears to be favorable and eco-friendly. In this case a high productivity is achieved with a natural wild strain, and thus the possibility for genetic improvisation is also very good.

Up-scaling and SSF in trays

SSF was performed in stationary trays analogous to conditions in a traditional Koji reactor as mentioned in "SSF in trays". The procedure was scaled up from 10 g wheat bran in 250-ml Erlenmeyer flasks to 1,000 g wheat bran in 80 × 40 × 4-cm trays (Table 1). By scaling up from flasks to stationary trays, activities of 154 IU/g DMB were reproducibly obtained. These results are therefore encouraging for optimization under pilot scale conditions.

Production of accessory enzymes

The culture extract in addition to phytase consists of 133.2, 41.58, and 310.34 IU/g DMB of xylanase, cellulase, and amylase, respectively (Table 1). The supplementation of these enzymes along with phytase decreases viscosity, amount of loose droppings, and increases mass gain, feed conversion, and egg production [36]. The starch and non-starch fractions of feed are efficiently hydrolyzed by hydrolytic enzymes improving prebiotic functional properties [23].

Partial purification and characterization of phytase

The ammonium sulfate precipitation and desalting by Sephadex G-25 procedure resulted in 69% enzyme recovery with purification of 2.5 fold and specific activity of 49.83 IU/mg of protein. The highest phytase activity was observed at pH 6.0. The enzyme retained ca. 75% activity over a wide pH range, 2.0–9.5 (Table 1). The pH optima and pH stability profile of phytase determines its ability to act efficiently in the crop and stomach of the digestive tract of poultry [9]. Fungal phytase acts efficiently in stomach (pH 2–5) and needs reactivation to maintain activity in the crop (pH 4–5) of poultry and bacterial phytases act vice versa [17]. The catalytic efficiency of *A. niger* NCIM 563 phytase will be more in both the crop and stomach of poultry because it retains activity over a wide pH range (2–9.5) and will not require reactivation.

The maximum phytase activity was at 55°C and declined thereafter (Table 1). Phytases from various *Aspergilli* show optimum temperature in the range 40–65°C [32]. Thermostability studies are performed to predict the stability of phytase during the dry pelleting process employed presently for commercial phytase produced under submerged conditions. At 45°C the phytase enzyme exhibited 90% of its original activity after 60 min. At 60°C the enzyme exhibited 80% activity after 5 min and 20% activity after 60 min (Table 1). Phytase from *E. coli* (which is considered a candidate for commercial phytase) has also been reported to retain only 24% activity at 60°C for 1 h [10]. This solid state product is therefore more efficient and cost effective than *E. coli* phytase. Because it does not require downstreaming such as pelleting at high temperature and product formulation, the entire fermented product can be dried and ground and this also does not require high temperature. Therefore the step of pelleting at high temperature can be avoided and the dried product from food grade fungus can be sold as animal feed enzyme.

Phytase activity was moderately stimulated in the presence of 5 mM Ca^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+}, and Pb^{2+} and inhibited in the presence of 1 mM Hg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, and Ag^{2+} (Table 1). The enzyme retained 63 and 43% activity in the presence of Zn^{2+} and Cu^{2+} in contrast to most phytate-degrading enzymes that are greatly inhibited by Cu^{2+} and Zn^{2+} [14]. Actually the influence of zinc, iron, copper, and calcium is potentially significant from an applied perspective with respect to phytase in animal feed. Retention of phytase activity in the presence of Pb^{2+} (125% for 5 mM) and Ag^{2+} (50% for 1 mM) provides an opportunity for phytate hydrolysis in soils contaminated with heavy metals. The partially purified phytase when subjected to gel filtration chromatography on Sephacryl S-200 along with standard markers showed its native molecular weight 87 kDa (Table 1). The K_m and V_{max} were 0.156 mM and 220 μm/min/mg using a Lineweaver–Burk plot. These values fall well within the range previously reported for microbial phytases [35]. The partially purified phytase exhibited single band on non-denaturing PAGE and is detected by activity staining (Fig. 3).

Phytase exhibited high efficacy in phytate hydrolysis at different pH releasing 50, 562.8, 797.8, 470,776, and 733 mg inorganic phosphorus/kg soybean meal at pH 1.5, 2.0, 2.5, 3.5, 5.5, and 6.5, respectively, under emulated gastric conditions (Fig. 4). Phytase from *Yersinia rhodei* hydrolyzes phytate phosphorus only up to pH 5.5 [13]. But phytase from *A. niger* NCIM 563 was more resistant to pepsin and released more inorganic phosphorus from soybean meal under emulated gastric conditions over a much broader pH range. This determines the efficacy of *A. niger* NCIM 563 phytase and is exceptional.

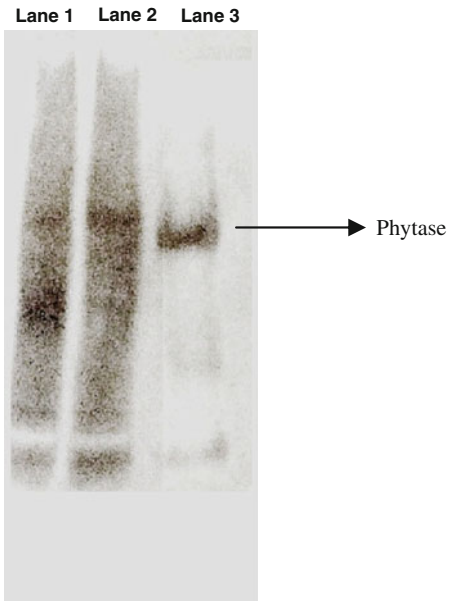

Fig. 3 Native gel electrophoresis (8%) of partially purified phytase from *A. niger* NCIM 563. *Lane 1* crude; *Lane 2* G25; *Lane 3* partially purified

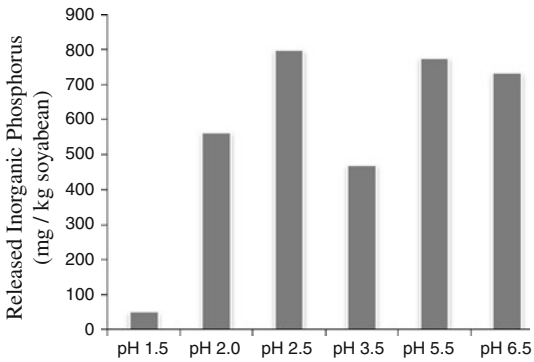

Fig. 4 Hydrolysis efficacy of phytate phosphorus in simulated gastric fluid at different pHs. Released inorganic phosphorus (mg/kg soybean) (*filled squares*). The degradation of phytate was measured after digestion at pH 1.5, 2.0, 2.5, 3.5, 5.5, and 6.5. Hydrolysis efficacy was calculated as the quantity of released inorganic phosphorus from soybean meal. All experiments are carried out in triplicates and their mean values are presented

Conclusion

The present work demonstrates that using response surface optimization employing PBD and BBD gave a high level of phytase production of 154 IU/g DMB along with accessory enzymes in SSF. Among the 11 chosen variables for optimization by PBD glucose, dextrin, $NaNO_3$, distilled water, and $MgSO_4 \cdot 7H_2O$ were found to influence phytase production more significantly. Some fungi are known to produce phytase and accessory enzymes by SSF but their low productivities are not comparable with the highest phytase productivity of 38,500 IU/kg/day by *A. niger*

NCIM 563 as shown by studies here. Tray fermentation studies for up-scaling also gave promising results from an industrial point of view. The process can be improved by using advanced bioreactors that provide accurate moisture and temperature control, as well as optimized O_2 mass transfer [8]. The partial characterization of phytase enzyme as studied here reveals some exclusive biochemical properties that bring out its potential for use as an animal feed additive. Among them are stability over broad pH range, high efficacy in hydrolyzing phytate phosphorus under emulated gastric conditions, and phytate hydrolysis of heavy metal contaminated soils. Instead of low yield, high cost Smf, SSF by *A. niger* NCIM 563 for phytase production emerges as a more efficient, less costly, and a more directly applicable process. All these factors corroborate the commercial potential and industrial application of phytase produced by *A. niger* NCIM 563 as a solid state culture product.

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