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Predicting vegetative inoculum performance to maximize phytase production in solid-state fermentation using response surface methodology

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Microbial phytase is used to reduce the environmental loading of phosphorus from animal production facilities. The limiting factors in the use of this enzyme in animal feeds can be overcome by solid-state fermentation (SSF), which is a promising technology for commercial enzyme production with lower production costs. Inoculum quality and the influence of inoculum quality on phytase production are important factors which need in-depth investigation before scaling-up of high-yielding fermentation process. A full factorial experimental design for 240 h with sampling at every 24 h was used to determine the effects of the treatments, inoculum age (plate and liquid culture), media composition and the duration of SSF on the production of fungal biomass and phytase in SSF systems using *Aspergillus niger*. The optimal treatment combination for maximal phytase production was determined by statistically comparing all treatments at each sampling time. Both 7- and 14-day plate cultures and M1+ medium composition with 72-h-old liquid inoculum treatments resulted in optimal phytase production at 144 h of SSF, which was the shortest duration observed for maximal phytase production. This resulted in maximal phytase production with a mean of 884±121 U/g substrate, while the maximal phytase production observed at 216 h of SSF (mean phytase activity of 1008±121 U/g substrate), with the same treatment combinations, was not statistically significant from that at 144 h of SSF. Phytase production was strongly growth-associated with younger inocula. The significant treatment variables, age of liquid inoculum and the duration of SSF, were used to predict the system response for phytase production using response surface methodology. From the response surface model, the optimal response of the experiment was predicted and the reliability of the prediction was checked with the verification experiment. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 161–170.

Keywords: phytase; solid-state fermentation; *Aspergillus niger*; statistical modeling; response surface methodology

Introduction

In animal diets, the supply of phosphorus, an essential mineral salt for animal growth and development, comes from either the feedstuffs or from inorganic feed phosphates added to the diets [17]. Although the total phosphorus content of the feedstuffs should be sufficient for the required phosphorus supplement, up to 80% of the total phosphorus present in most feedstuffs of plant origin exists in the form of phytic acid phosphorus. Monogastric animals, such as swine and poultry, lack sufficient amounts of intrinsic phytases to hydrolyze the phytic acid complexes [2,15]. Phytic acid has antinutritive properties and forms complexes with protein and multivalent cations such as Zn^{2+}, Ca^{2+} and Fe^{3+} and reduces their bioavailability [14]. Phytic acid also inhibits a number of nutritionally important enzymes *in vivo* [7]. These nutritional impediments result in the release of undigested phytate phosphorus in the feces and urine.

Phosphorus in the environment accelerates eutrophication of fresh waters and is the main problem in surface water quality, resulting in restricted water use for fisheries, recreation, industry and drinking [4,20]. In Europe, stringent environmental legislation controls the amount of phosphorus that can be released to the environment, and the US is also beginning to face similar regulations [2]. Many researchers currently state that due to economic and environmental significance of the problem, any method that can improve the phosphorus digestibility in feedstuffs or reduce the amount of phytate in plants would be beneficial [2,17].

The phytases (*myo*-inositol hexakisphosphate 3- and 6-phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) in the subfamily of the histidine acid phosphatases [12] convert phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) to inositol and phosphoric acid (Figure 1) [21], making phosphorus available for bioabsorption [10]. Supplementation of microbial phytase to animal diets alters the phytic acid complexes and also increases the bioavailability of proteins and essential minerals, providing growth performance equivalent or better than those with phosphate supplementation, and also reduces the amount of phosphorus in animal manure [21]. If phytase were added to the diets of all monogastric animals reared in the US, the value of the phosphorus released would be US1.68×10^8 per year, and it would reduce environmental loading of phosphorus by 8.23×10^7 kg [21]. The FDA has approved a generally-recognized-as-safe (GRAS) petition for use of phytase in food, and phytase has been marketed as a feed additive in the US since 1996 [21].

Microbial phytases are produced commercially by submerged fermentation (SmF). However, the high cost of enzyme was

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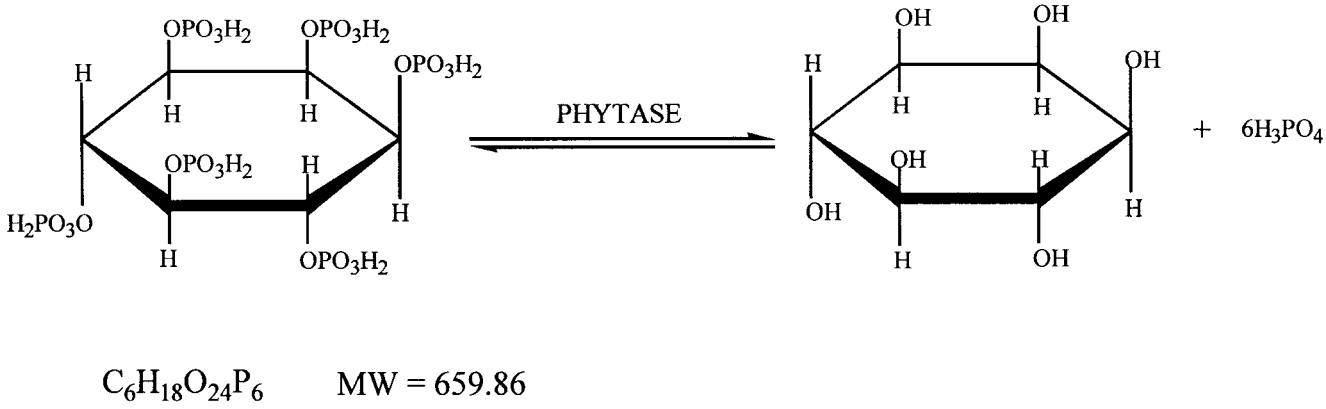

Figure 1 Structure showing the enzymatic hydrolysis of phytic acid to inositol and phosphoric acid.

cited as the limiting factor in using the enzyme in animal diets [8,21]. Extracellular hydrolytic enzymes and other metabolites are produced in high concentrations in solid-state fermentation (SSF). SSF refers to the growth of microorganisms on solid substrates without the presence of free liquid [3]. SSF is a promising technology for commercial enzyme production because it can use unrefined agro-industrial waste products as substrate, in general, uses a less expensive process, requires lower capital investment and operational costs and results in a higher volumetric productivity over SmF. The entire fermented product can be dried, ground and sold as animal feed, resulting in less waste and less downstream processing.

The scaling-up and optimization of SSF processes necessary for commercial production, however, need intensive research [19]. Inoculum quality contributes substantially to successful and high-yielding fermentations and therefore must be thoroughly understood. An understanding of microbial growth kinetics on solid substrates is necessary for the design, scale-up, operation and optimization of SSF bioreactors. Phytase production by SSF has been reported using canola meal as substrate by *Rhizopus oligosporus*, *Aspergillus niger* and *A. ficuum* [14]. However, published reports are insufficient regarding the influence of vegetative inoculum culture conditions on SSF because most of the studies used spore suspensions. A previous study on phytase production using this strain of *A. niger* on wheat bran reported that a vegetative inoculum is needed for the SSF process [16]. This strain needs a vegetative inoculum because spore production is minimal.

Another aspect of phytase production that needs investigation is determining whether or not phytase is growth-associated. Phytase production in SSF using *A. carbonarius* with canola meal as substrate was reported to be growth-associated [1]. However, there are no reports available on the effect of culture conditions on growth and its relation to phytase production in SSF systems.

The objective of this study was to determine the effect of culturing conditions of *A. niger* for the vegetative inoculum on phytase production and growth in SSF. Specifically, we investigated the effect of plate age for the initial inoculum, the influence of wheat bran in the liquid culture media and the age of liquid inoculum. The interaction of these factors with duration of the SSF was also investigated. Phytase and biomass measurements are compared to determine if phytase is growth-associated.

Materials and methods

Microorganism and culture conditions

A phytase-producing strain of *A. niger* (provided by Alltech, Nicholasville, KY, USA) was used. Culture maintenance included a bimonthly subculture from a molasses agar plate to a potato dextrose agar (PDA) plate and storage at 25°C. Inoculation was accomplished by cutting a 5×5 mm^2 agar block from the growing edge of the culture and transferring it to the center of the new plate. PDA plates were incubated for 7 and 14 days, respectively, and used as the plate culture inoculum for liquid inoculum preparation for SSF.

Liquid inoculum preparation for SSF

The standard synthetic liquid medium (M1−) contained (g/l): corn starch 28; glucose 5; peptone 18; KCl 0.5; $MgSO_4{\cdot}7H_2O$ 1.5; KH_2PO_4 1; $CaCl_2{\cdot}2H_2O$ 2. When complex medium (M1+) was used, 20 g/l of wheat bran was included. One hundred milliliters of the medium was dispensed into 250-ml Erlenmeyer flasks and sterilized at 121°C for 20 min. When wheat bran was included in the medium, the bran was sterilized separately and added to the sterile medium aseptically prior to inoculation. The pH of the media after sterilization was 5.0–5.3. Addition of wheat bran to the medium increased the initial pH of 5.5–5.8. The pH was not adjusted during the study.

Table 1 Experimental factor levels for the full factorial experimental design to optimize process parameters for the production of phytase by *A. niger* in SSF systems

Class variables	Levels	Values	Units
Replicates	2	1, 2	
Age of liquid culture (X_1)	4	24, 48, 72, 96	h
Duration of SSF (X_2)	10	24, 48, 72, 96, 120, 144, 168, 192, 216, 240	h
Age of plate culture (X_3)	2	7, 14	days
Media Composition (X_4)	2	Standard synthetic medium (M1−) and complex medium (M1+) (standard synthetic medium with wheat bran)	

Flasks were inoculated with 7- or 14-day fungal cultures grown on a PDA plate by aseptically transferring a block of mycelium and spores (5×5 mm^2 area) of the culture into the flask which were incubated in an environmental shaker at 200 rpm and 30°C for up to 96 h.

SSF

SSF was carried out in 250-ml flasks containing air-dried solid substrates (3.5 g of wheat bran and 1.5 g of full-fat soybean flour). The flasks with substrate were sterilized at 121°C for 20 min prior to inoculation. Moisture content was adjusted to 53% (wet basis)

Table 2 Data for the full factorial experiment evaluating the effects of inoculum age (plate culture in days and liquid culture in hours), media composition (M1− for standard synthetic medium and M1+ for complex medium) and duration of fermentation on phytase activity (U/g substrate) and glucosamine content (g/g substrate) by *A. niger* in SSF

[Age of the liquid culture]

Duration of SSF (h)	Inoculum from 24-h liquid culture		Inoculum from 48-h liquid culture		Inoculum from 72-h liquid culture		Inoculum from 96-h liquid culture	
	M1− medium	M1+ medium	M1− medium	M1+ medium	M1− medium	M1+ medium	M1− medium	M1+ medium
Ages of the PDA plate culture — 7 days								
[*Phytase activity (U/g substrate)*]								
24	0	2	7	14	6	5	4	1
48	22	36	115	156	62	70	63	123
72	194	223	274	366	302	416	187	392
96	407	442	456	610	454	557	333	629
120	577	608	583	695	644	708	433	733
144	699	690	693	781	705	846	639	753
168	763	856	766	832	809	855	717	777
192	787	873	802	868	824	844	863	999
216	889	746	823	854	993	1028	746	977
240	621	593	786	860	753	708	692	963
[*Glucosamine content (g/g substrate)*]								
24	0.002	0.003	0.002	0.004	0.007	0.005	0.004	0.005
48	0.004	0.009	0.010	0.013	0.014	0.016	0.010	0.013
72	0.010	0.014	0.015	0.018	0.019	0.020	0.011	0.017
96	0.017	0.017	0.020	0.022	0.022	0.023	0.027	0.039
120	0.019	0.018	0.023	0.025	0.032	0.029	0.032	0.044
144	0.023	0.020	0.026	0.029	0.026	0.031	0.032	0.032
168	0.026	0.024	0.030	0.032	0.029	0.030	0.031	0.028
192	0.025	0.027	0.030	0.032	0.029	0.032	0.025	0.033
216	0.029	0.024	0.029	0.033	0.035	0.030	0.017	0.032
240	0.023	0.020	0.028	0.035	0.039	0.030	0.017	0.014
Age of the PDA plate culture — 14 days								
[*Phytase activity (U/g substrate)*]								
24	1	2	8	6	15	13	13	24
48	61	165	109	152	212	164	218	279
72	326	387	298	368	573	678	433	481
96	503	536	443	500	724	736	513	500
120	545	648	556	608	758	805	600	703
144	784	752	633	686	747	868	775	749
168	640	668	710	793	905	874	755	899
192	741	775	824	934	895	865	862	945
216	878	756	809	844	884	988	765	1111
240	900	742	714	784	858	878	685	998
[*Glucosamine content (g/g substrate)*]								
24	0.004	0.006	0.004	0.005	0.006	0.008	0.011	0.010
48	0.011	0.011	0.013	0.012	0.021	0.024	0.018	0.022
72	0.020	0.020	0.024	0.021	0.029	0.030	0.025	0.031
96	0.028	0.028	0.032	0.032	0.027	0.029	0.030	0.038
120	0.032	0.029	0.030	0.032	0.032	0.032	0.032	0.035
144	0.035	0.032	0.031	0.034	0.034	0.033	0.029	0.030
168	0.034	0.026	0.021	0.028	0.033	0.035	0.027	0.026
192	0.030	0.025	0.013	0.021	0.028	0.031	0.026	0.026
216	0.024	0.019	0.010	0.018	0.027	0.029	0.018	0.018
240	0.018	0.015	0.008	0.007	0.013	0.012	0.014	0.012

Numbers represent the average of two replications with two subsamples.

by aseptically adding 2.9 ml of sterile water and 3 ml of liquid inoculum. The flasks were incubated at 30°C for 240 h.

Two flasks from each treatment were harvested every 24 h; 0.5 g of the fermented sample was used for glucosamine analysis and the rest of the sample was used for enzyme extraction. The extracellular enzyme was extracted by adding 20 ml distilled water and 0.1% (v/v) Tween 80 per gram of initial solid media and homogenizing the suspension, then shaking it at 200 rpm for 1 h at 30°C. The homogenized suspension was filtered through Whatman no. 1 paper and the clear filtrate was used as the crude enzyme extract. Values obtained for the two flasks from each replication were averaged and used for statistical analysis.

Analytical methods

Phytase activity: Phytase activity was determined after enzymatic hydrolysis of sodium phytate under controlled conditions and measurement of the amount of *ortho*-phosphate released [9]. One unit of the enzyme activity was defined as the amount of phytase required to liberate 1μmol of inorganic phosphate per min under standard assay conditions.

Biomass in solid culture: In SSF, fungal mycelia are closely bound to the solid matrix [6], which prevented direct recovery and determination of biomass from the solid substrate culture media. Studies on SSF rely on indirect methods of biomass determination [11]. Glucosamine is an essential and stable component of mycelial cell walls and is used as an efficient parameter for the estimation of the total sum of the growing mycelium because changes in glucosamine are believed to correspond to development of the mycelium. But the values cannot be considered as a direct quantitative measurement of the mycelial weight [18]. Glucosamine was released from the biomass by hydrolysis according to the method of Sakurai *et al.* [18]. Results of glucosamine measurements were corrected for the background level of glucosamine present in the raw solid substrate and are expressed in grams glucosamine per gram of fermented substrate.

Experimental design and statistical analysis

A full factorial experimental design was used for the experiment (Table 1). PROC GLM in SAS® Software (SAS Institute, Cary, NC, USA) was used to evaluate the data, main effects and treatment combinations (media, plate age, liquid inoculum age and duration of SSF). The dependent variables quantified were phytase production under SSF (U/g substrate) and glucosamine content under SSF (g/g substrate).

Data from the full factorial model were subjected to a second-order multiple regression analysis using least squares regression methodology to obtain the parameters for the response surface (PROC RSREG in SAS® Software). The general form of the polynomial model for two factors is given in the following equation (Equation 1) where Y_i is the response variable; X_1 and X_2 are the factor variables; β terms are the calculated parameter estimates for each main effect, interaction and quadratic effect; and ε is the error:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2 + \varepsilon \qquad (1)$$

The optimum for the liquid inoculum age and duration of SSF for phytase production was determined from the response surface. Contour plots were developed from the fitted quadratic polynomial equation obtained from the PROC RSREG (SAS® Software) analysis by holding the factor with the least effect on the response surface constant and changing the other two variables.

PROC REG (SAS® Software) was used to fit the model by least squares and to predict the relationship between phytase activity and glucosamine content in SSF. All data presented are the mean values of two replications with two subsamples.

Verification studies

Verification experiments were conducted at the optimal conditions selected from the response surface to check the model. Experiments were conducted in two replications with two subsamples.

Results and discussion

Optimal inoculum culture conditions for phytase production in SSF

Treatments and their means for phytase production are presented in Table 2 for the experiment investigating the effects of

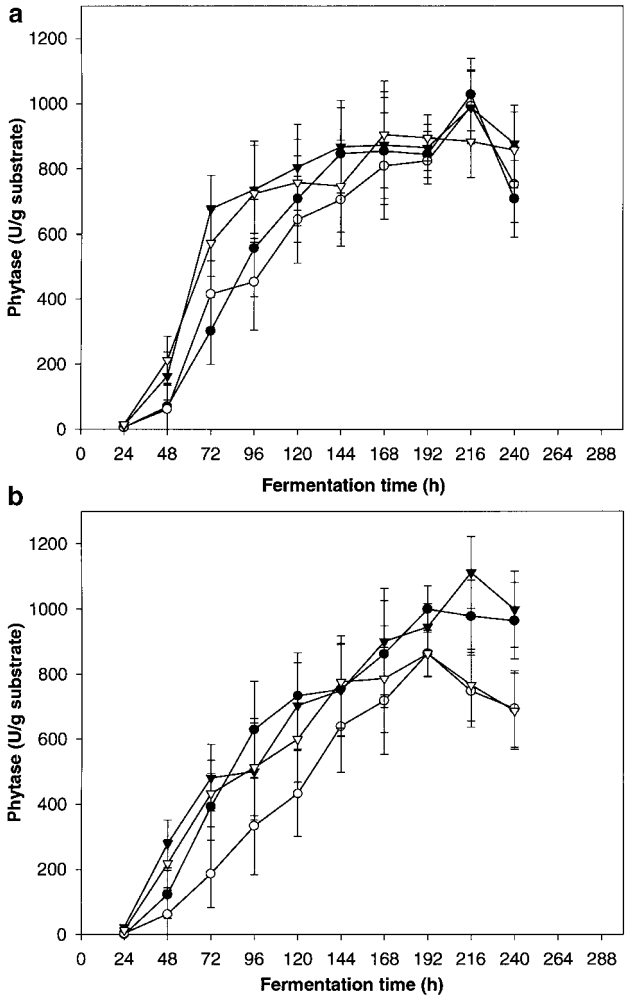

Figure 2 (a) Mean phytase production obtained in a SSF inoculated from 72-h-old liquid culture. Treatments: (●) 7-day plate and M1 + medium; (○) 7-day plate and M1 − medium; (▼) 14 day plate and M1 + medium; (▽) 14-day plate and M1 − medium). (b) Mean phytase production obtained in a SSF inoculated from a 96-h-old liquid culture. Treatments: (●) 7-day plate and M1 + medium; (○) 7-day plate and M1 − medium; (▼) 14-day plate and M1 + medium; (▽) 14-day plate and M1 − medium.

Table 3 Treatment combinations that gave maximal phytase production by *A. niger* on wheat bran/soy meal in SSF systems

Treatment combination	Optimal fermentation time (h) for maximal phytase production	Mean phytase activity (U/g substrate) (MSE=121 U/g substrate)
7-day plate culture, M1+ medium liquid inoculum treatments		
24-h-old liquid inoculum	168–192	862
48-h-old liquid inoculum	168–240	849
72-h-old liquid inoculum	144–216	879
96-h-old liquid inoculum	168–240	929
14-day plate culture, M1+ medium liquid inoculum treatments		
24-h-old liquid inoculum	192–216	766
48-h-old liquid inoculum	192–240	854
72-h-old liquid inoculum	144–240	888
96-h-old liquid inoculum	168–240	970
7-day plate culture, M1− medium liquid inoculum treatments		
24-h-old liquid inoculum	192–216	838
48-h-old liquid inoculum	192–240	804
72-h-old liquid inoculum	192–216	909
96-h-old liquid inoculum	192	863
14-day plate culture, M1− medium liquid inoculum treatments		
24-h-old liquid inoculum	216–240	889
48-h-old liquid inoculum	192–240	817
72-h-old liquid inoculum	168–240	889
96-h-old liquid inoculum	192	862

inoculum culture conditions (age of plate culture, medium composition and the age of liquid inoculum) on the optimal duration of SSF. Phytase production from representative treatments (72- and 96-h liquid inoculum) of the SSF experiment is shown in Figure 2a and b. All treatments were statistically compared at each sampling time and the optimal treatment combination for maximal phytase production was determined. The treatment combinations that gave maximal phytase production in SSF are given in Table 3. Phytase production from the treatment combinations listed in Table 3 was not statistically different (significance level of 5%).

The set of optimal treatment combinations was compared to see if trends regarding the treatment variables could be determined. The effect of plate age (primary seed culture) on phytase yield was evaluated by comparing phytase production at each treatment level of media composition and liquid culture age (Table 3). Comparing the 7-day plate culture, M1+ medium with the 14-day plate culture, M1+ medium, 24- and 48-h-old liquid inoculum, the duration of SSF for maximal phytase production was shorter (by 24 h) for the 7-day plate. Both 7- and 14-day plates (M1+ medium) responded identically to the 72-h liquid inoculum treatments with optimal phytase production observed at 144–216 h SSF. The fermentation for maximal phytase production was shortened by 24 h compared to other liquid inoculum treatments. Both plate cultures, M1+ medium, 72-h liquid inoculum treatments resulted in maximal enzyme production at the shortest duration of SSF (at 144 h of SSF) compared to any other treatment. This resulted in a mean phytase activity of 884±121 U/g substrate with the shortest duration of SSF. When the liquid inoculum was older (96 h), both plate cultures, with M1+ medium, responded with the same trend with maximal phytase production at 168–240 h of SSF. This resulted in a maximal phytase production with a mean of 950±121 U/g substrate. However, phytase yield at these treatments was not statistically different from that observed at the shortest duration of SSF. Therefore, older liquid inocula prepared using M1+ medium appear to need a longer fermentation time for product optimization.

Within the M1− medium treatment when comparing both plate ages, 24-h-old liquid inoculum produced maximal phytase 24 h earlier (at 192 h SSF) with 7-day plate culture, compared to 216 h with the 14-day plate culture. With the 48-h-old liquid inoculum, maximal phytase production (mean activity 811±121 U/g substrate) was obtained at 192–240 h with both plate cultures. The 72-h-old liquid inoculum, 14-day plate culture resulted in maximal phytase production (mean activity 874±121 U/g substrate) in shorter SSF time (168 h) compared to any

Table 4 Regression model showing the relationship between the dependent variables, phytase activity (y) (U/g substrate) and glucosamine content (x) (g/g substrate), and the effect of treatments (age of plate and liquid culture, and media composition) in SSF system

Treatments (SSF flasks inoculated with liquid culture from)	Age of liquid culture (h)	Regression coefficient (R^2)	Regression equation ($y=ax+b$, where y is phytase activity; and x is glucosamine)	Parameter estimate		Parameter estimate	
				a	Pr > F	b	Pr > F
7-day plate, M1+ medium	24	0.9296	$y=42{,}236x-236.45$	42,236	0.0001	−236.45	0.0160
	48	0.9638	$y=31{,}276x-140.78$	31,276	0.0001	−140.78	0.0333
	72	0.8729	$y=36{,}697x-291.71$	36,697	0.0001	−291.71	0.0516
	96	0.6196	$y=31{,}399x+26.94$	31,399	0.0069	+26.94	0.9024
7-day plate, M1− medium	24	0.9871	$y=33{,}600x-107.18$	33,600	0.0001	−107.18	0.0041
	48	0.9816	$y=32{,}496x-148.68$	32,496	0.0001	−148.68	0.0031
	72	0.8379	$y=30{,}939x-216.74$	30,939	0.0002	−216.74	0.1297
	96	0.7974	$y=38{,}944x-139.33$	38,944	0.0005	−139.33	0.2715
14-day plate, M1+ medium	24	0.7274	$y=46{,}115x-226.00$	46,115	0.0017	−226.00	0.2700
	48	0.6074	$y=41{,}498x-191.91$	41,498	0.0079	−191.91	0.4192
	72	0.4861	$y=26{,}350x+49.24$	26,350	0.0250	+49.24	0.8457
	96	0.1915	$y=18{,}952x+274.70$	18,952	0.2060	+274.70	0.3937
14-day plate, M1− medium	24	0.7012	$y=35{,}515x-83.80$	35,515	0.0025	−83.80	0.6431
	48	0.7421	$y=30{,}705x-97.56$	30,705	0.0014	−97.56	0.4933
	72	0.4749	$y=32{,}505x-48.27$	32,505	0.0275	−48.27	0.8639
	96	0.5872	$y=31{,}307x-61.11$	31,307	0.0097	−61.11	0.7606

other liquid inoculum treatments. Both plate cultures, when inoculated with 96-h-old liquid inoculum, produced maximal phytase (mean activity 863±121 U/g substrate) at 192 h of SSF.

In summary, when M1+ medium was used, the 7-day plate culture was more efficient in producing maximal phytase at shorter SSF times with younger liquid inoculum. The 72-h-old liquid inoculum from both (7- or 14-day) plates yielded maximal phytase at 144 h, which was the shortest duration SSF observed. This suggests that the overall performance of fermentation can be shortened using 72-h liquid inoculum. The increase in required SSF time using 96-h liquid inoculum might be due to the aging of the fungal cells in the liquid culture, resulting in less active cells on the solid substrate, which leads to a longer lag phase.

The M1− medium, 7-day plate culture with all liquid inoculum treatments showed the same trend for maximal phytase production at longer SSF times (192 h). With 48- or 96-h liquid inoculum from 14-day plate cultures, M1− medium showed the same trend for maximal phytase production with an optimum at 192 h. With 14-day plate cultures, M1− medium when inoculated with younger liquid inoculum (24 h) maximal phytase production

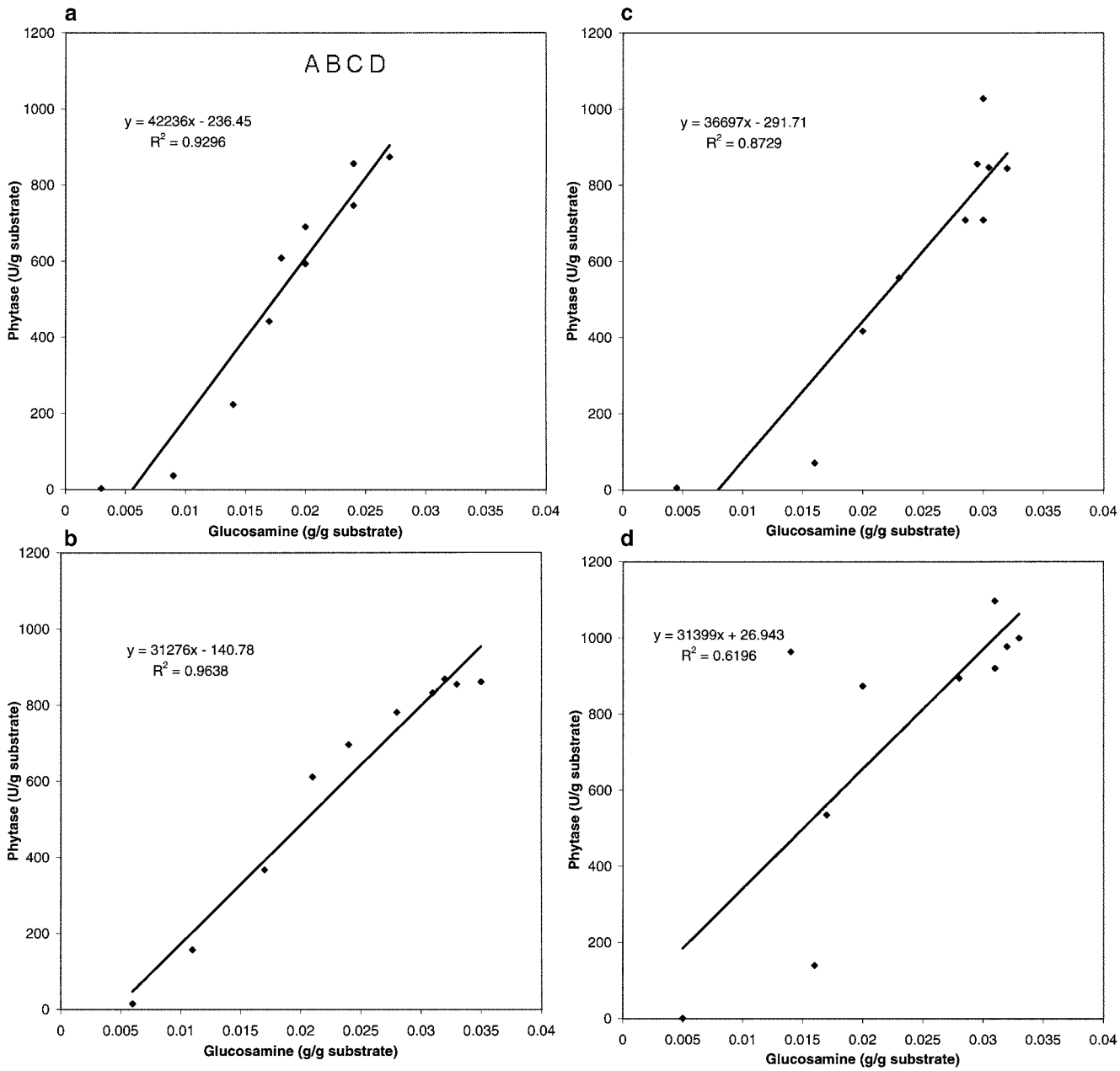

Figure 3 (a) Correlation between phytase production (U/g substrate) and glucosamine content (g/g substrate) in SSFs in M1+ medium inoculated from a 7-day plate culture and a 24-h liquid culture. (b) Correlation between phytase production (U/g substrate) and glucosamine content (g/g substrate) in SSFs inoculated from M1+ medium, 7-day plate culture and 48-h liquid culture. (c) Correlation between phytase production (U/g substrate) and glucosamine content (g/g substrate) in SSFs inoculated from M1+ medium, 7-day plate culture and 72-h liquid culture. (d) Correlation between phytase production (U/g substrate) and glucosamine content (g/g substrate) in SSFs inoculated from M1+ medium, 7-day plate culture and 96-h liquid culture.

was accomplished at longer SSF times (216–240 h). With 72-h liquid inoculum, maximal phytase production was attained with 14-day plate at 168 h of SSF, the shortest SSF time for the M1− medium treatments. This resulted in maximal phytase production with a mean of 874±121 U/g substrate.

The effect of media composition at each plate culture age and liquid inoculum age on phytase production was compared to investigate the influence of media composition on fermentation performance (Table 3). With a 7-day plate culture, M1+ medium using younger liquid cultures (24, 48 h) resulted in maximal phytase at 168–240 h SSF. However, when M1− medium was used, maximal phytase production was observed at 192–240 h. The duration of SSF for maximal phytase production was shortened by 24 h when M1+ medium was used. With a treatment combination of 72-h-old liquid inoculum from 7-day plate culture, the use of M1+ medium shortened the optimal SSF time by 48 h compared to treatments using M1− medium. When inoculated with 96-h-old liquid culture, M1+ medium, 7-day plate culture, maximal phytase was attained at 168 h of SSF, compared to 192 h with M1− medium.

When a 14-day plate culture was used as the inoculum, with 24-h-old liquid inoculum, M1+ medium resulted in maximal phytase production at 192–216 h, compared to 216–240 h with M1− medium. With both media, when 48-h-old liquid inoculum was used, maximal production occurred at 192–240 h of SSF. The duration of SSF was shortened by 24 h when 14-day plate culture, M1+ medium was used for both the 72- and 96-h-old inocula, compared to M1− medium.

Comparison of media composition on phytase production suggests that with either the 7- or 14-day plate culture, use of M1+ medium will result in maximal phytase production in a shorter time. Thus, it will be advantageous to use M1+ medium for liquid inoculum preparation (secondary seed culture) to reduce production time. Addition of wheat bran to liquid medium influenced phytase yield by shortening the SSF phase. In the presence of wheat bran (complex medium), this strain of *A. niger* grew as a mixture of fine pellets, clumps and mycelial trees, and in the absence of wheat bran (standard synthetic medium), they grew as large pellets in liquid culture media (not shown). The hyphal mode of growth in the presence of wheat bran in complex medium (M1+ medium) leads to more rapid colonization of the solid substrate and the more efficient utilization of available nutrients. This might be the reason for maximal phytase production in shorter fermentation time when the liquid inoculum was prepared using M1+ medium.

Information regarding the influence of the age of plate culture as inoculum and its influence on performance of the fermentation process is of concern for successful industrial applications. Industrial fermentation processes use primary, secondary, as well as tertiary and quaternary seed cultures as inoculum, and performance of the seed culture depends on the plate culture used. Higher phytase activity in a shorter SSF time with younger liquid inoculum was observed when 7-day plate culture, M1+ medium was used as primary culture. From statistical analysis, the age of liquid inoculum treatment and duration of SSF have a strong influence on the fermentation performance. Trends in these variables were further analyzed using response surface methodology.

Treatments for glucosamine production are presented in Table 2. The treatments were statistically compared at each sampling time to find the optimal treatment combinations for glucosamine production during SSF. Glucosamine production was optimal from 144 to 240 h when 24-h-old liquid culture from 7-day plate culture with either medium were used. All other treatment combinations using 7-day plate culture resulted in optimal glucosamine production from 96 to 192 h regardless of the medium. With 14-day plate cultures, younger liquid cultures (24 and 48 h) resulted in higher glucosamine production at 96 h with either media. All the other treatment combinations using 14-day plate culture resulted in an optimal glucosamine production at 72 h of SSF.

Relationship between phytase activity and growth

Linear regressions were performed to determine if phytase production could be predicted from glucosamine content. The resulting equations are given in Table 4. The R^2 showed that phytase is strongly linearly related to glucosamine when the liquid culture inoculum is younger. A strong correlation (R^2 of 0.93–0.99) between phytase activity and glucosamine content was observed with both media when they were inoculated with 24- to 48-h-old liquid culture from a 7-day plate culture. With 72-h-old liquid culture, R^2 values of 0.87 for M1+ medium and 0.84 for M1− medium were observed; with 96-h-old liquid culture, R^2 values of 0.62 for M1+ medium and 0.80 for M1− medium were observed (Table 4). Representative figures showing the relationship between phytase activity and glucosamine content and the effect of each liquid inoculum treatments when inoculated from a 7-day plate culture, M1+ medium are shown (Figure 3a–d). With a 14-day plate culture and M1+ medium, the correlation between phytase activity and glucosamine content was significant with 24-, 48- and 72-h-old liquid cultures. However, the relationship was not as strong as that of 7-day culture, and with 96-h liquid culture, the relationship was not significant. With a 14-day plate culture and

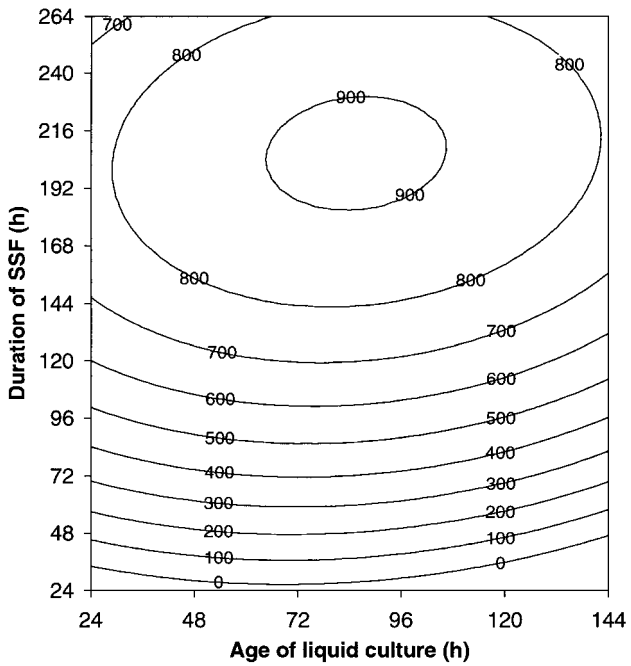

Figure 4 Contour plot showing the dependence of phytase activity (U/g substrate) by *A. niger* in SSF (inoculated with liquid culture from a 7-day plate culture from M1+ medium) on the duration of fermentation [age of liquid culture (h) *versus* duration of SSF (h)] (data from the response surface model).

Table 5 Prediction equations obtained for the dependent variable, phytase activity (Y), in SSF systems

Treatments (SSF inoculated with liquid culture from)	Explanatory equation (where Y=phytase activity; X_1=age of liquid culture; and X_2=duration of SSF)	R^2	Pr>F
7-day plate, M1+ medium phytase (U/g substrate)	$Y=-450.79+4.84X_1+11.23X_2-0.036X_1^2-0.029X_2^2+0.0067X_1X_2$	0.8781	0.0003
14-day plate, M1+ medium phytase (U/g substrate)	$Y=-303.05+2.91X_1+9.89X_2-0.022X_1^2-0.026X_2^2+0.014X_1X_2$	0.8969	0.0053
7-day plate, M1− medium phytase (U/g substrate)	$Y=-431.02+6.51X_1+9.50X_2-0.057X_1^2-0.0204X_2^2-0.00084X_1X_2$	0.9401	0.0000
14-day plate, M1− medium phytase (U/g substrate)	$Y=-455.995+5.90X_1+10.77X_2-0.0308X_1^2-0.0248X_2^2-0.0089X_1X_2$	0.8657	0.0001

M1− medium, the relationship between phytase activity and glucosamine content was significant, but not as strong as that of a 7-day culture (Table 4).

Phytase production was strongly growth-associated in SSF when cultures were inoculated with liquid cultures from a 7-day plate culture. This might be explained with the growth kinetics of the fungal mycelia on solid substrates. In SSF, growth is characterized by apical hyphal extension on the surface of the solid matrix, but the growth kinetics of the mycelia depends on the availability of nutrients and the geometric configuration of the matrix [13]. The fungal mycelia grow on the solid substrate mainly by surface adhesion. The cellular components of the mycelium or even the single hyphal strands are in different physiological states; the hyphal tips contain actively growing cells while the non-growing part may contain slowly metabolizing or even dead cells [13]. With younger inoculum, the fungi might be in the active growth phase, which contributes to the active utilization of the substrate and further product formation. Factors such as the formation of vacuoles in the cytoplasm, the increasing age and sizes of the cells and accumulation of toxic waste during fermentation lead to mechanical damage of hyphae and inactivation of cells [5]. From the experimental results, it is clear that the glucosamine content of the growing cells of the mycelium is variable. Glucosamine content is affected by culture conditions such as age and growth phase of the culture. Chitin content of the fungal hyphae may change with the age of the culture [5], which may lead to variation of glucosamine measurements between cultures at different growth stages. Smits *et al.* [19] also observed that glucosamine content of the biomass changes with the age and physical state of the fungi.

Response surface methodology

Response surface methodology was used to fit a model to predict the optimal response. The data were modeled for each plate age and medium composition combinations separately. Controllable process variables (age of liquid inoculum and duration of SSF) were included in the response surface model. The analysis of variance (ANOVA) for the response surface model that explains the response of phytase production in SSF when inoculated from a 7-day plate culture, M1+ medium gives a determination coefficient (R^2) of 0.88. Linear and quadratic interactions were highly significant (Pr>F=0.0001). The term corresponding to the cross-product interaction between the age of liquid inoculum and duration of SSF was not significant (Pr>F=0.4546). The lack of fit tests was not significant (Pr>F=0.9960). The individual effect of factor variables on phytase production was significant with the age of liquid inoculum (X_1) at the 7% level and duration of SSF (X_2) at the 5% level. Figure 4 shows the predicted response of phytase production on the age of liquid inoculum and duration of SSF for a 7-day plate culture, M1+ medium based on the explanatory equation given in Table 5. Canonical analysis of the response surface resulted in prediction of the maximal phytase production of 916 U/g substrate when a liquid culture 86-h old is used to inoculate SSF flasks that are incubated for 207 h (Table 6).

The ANOVA for the response surface model that explains the response of phytase production in SSF, when inoculated from liquid culture from 14-day plate culture, M1+ medium, gives a determination coefficient (R^2) of 0.90. Linear and quadratic terms were highly significant (Pr>F=0.0001) and the cross-product interactions had a moderate effect (significant at the 8% level). The lack of fit test was not significant (Pr>F=0.8630). The individual effect of factor variables, age of liquid inoculum (X_1) and duration of SSF (X_2) significantly (Pr>F=0.0001) affect phytase production. Figure 5 shows the predicted response of phytase production on the age of liquid culture and duration of SSF based on the explanatory equation given in Table 5. The contour plot clearly shows the strong impact of the factor variables on the response surface with a maximal phytase activity of 1011 U/g substrate at a liquid culture age of 133.79 h inoculated into SSF and incubated for 226.45 h (Table 6).

Statistical analysis of the 7- and 14-day plates using M1− medium as liquid inoculum treatments, followed with SSF, resulted in maximal phytase production with shorter liquid culture ages and

Table 6 Canonical analysis of response surface: optimal values obtained for the dependent variable, phytase activity, in the SSF system (X_1=age of liquid culture; X_2=duration of SSF)

Treatments (SSF inoculated with liquid culture from)	Dependent variable	Critical value				Predicted value	Stationary point
		Coded		Uncoded			
		X_1	X_2	Age of liquid culture (h)	Duration of SSF (h)		
7-day plate, M1+ medium	Phytase (U/g substrate)	0.711	0.690	85.58	206.54	916.09	Maximum
14-day plate, M1+ medium	Phytase (U/g substrate)	2.05	0.875	133.79	226.45	1011.17	Maximum
7-day plate, M1− medium	Phytase (U/g substrate)	−0.117	0.922	55.77	231.56	850.53	Maximum
14-day plate, M1− medium	Phytase (U/g substrate)	0.166	0.677	65.96	205.11	842.86	Maximum

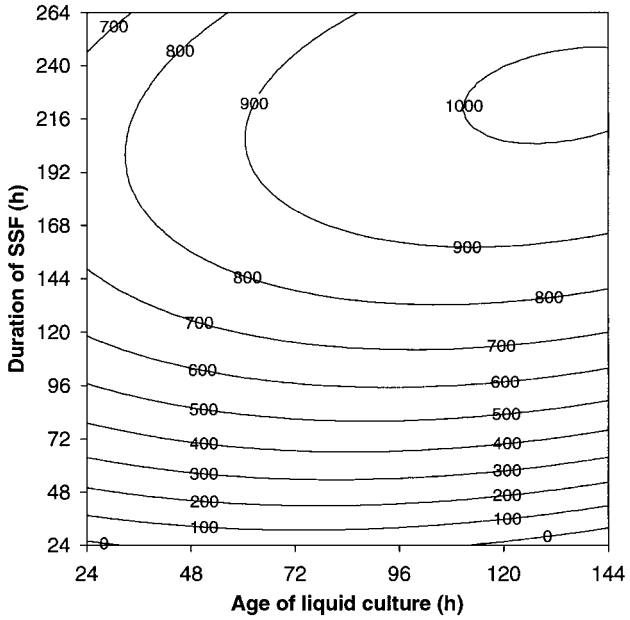

Figure 5 Contour plot showing the dependence of phytase activity (U/g substrate) by *A. niger* in SSF (inoculated with liquid culture from a 14-day plate culture from M1 + medium) on the duration of fermentation [age of liquid culture (h) *versus* duration of SSF (h)] (data from the response surface model).

longer SSF times compared to M1+ medium, except that lower phytase production was obtained. The resulting explanatory equations are listed in Table 5, and the predicted maximum values, along with the required factor variables to achieve these values, are included in Table 6. The response surfaces were similar in shape to that using M1+ medium.

Table 6 presents the canonical analysis of the response surface with optimal values obtained for dependent variables in the SSF process. Results of the predicted response surface showed a maximal phytase production of 1011 U/g substrate when M1+ medium was inoculated with a liquid culture of 133.79-h, 14-day plate, and incubated for 226.45 h in SSF. With standard synthetic medium, the highest phytase can be attained at a shorter age of liquid culture (55–65 h), but yielded less phytase. However using a 7-day plate and M1− medium, the incubation time for SSF was longer (231.56 h) and the phytase yield was 7.7% lower compared to M1+ medium. With a 14-day plate and M1− medium, the incubation time for SSF was shorter (205.11 h) but the phytase yield was 20% lower compared to M1+ medium. If production turn-around is taken into consideration along with phytase yield, the 7-day plate, complex medium will give optimal phytase production of 916 U/g substrate at a shorter liquid culture age of 85.58 h and a solid-state cultivation time of 206.54 h.

Table 7 presents the verification model with experimental data as well as the predicted values from the response surface model under the selected operational conditions. Phytase production under the selected operational conditions for the verification model for all treatments was within the range of the predicted value from the response surface model. The response surface model was useful for predicting and optimizing the system response in the fermentation process.

Conclusions

Statistically evaluating all treatment combinations allowed the optimal fermentation strategy to be determined. Duration of SSF can be shortened to 144 h for maximal phytase production using 72-h-old liquid inoculum from 7- to 14-day plates, M1+ medium treatments. At this time period, mean phytase activity of 884±121 U/g substrate could be obtained. This maximal yield at 144 h of SSF was not statistically different (5% level) from that observed at 216 h of SSF with the same treatment combinations with a mean phytase activity of 1008±121 U/g substrate. In general, higher phytase activity can be achieved in less time when younger liquid inoculum from a 7-day plate culture, M1+ medium was used. Phytase production was also strongly growth-associated in SSF when inoculated with a younger liquid culture from a 7-day plate culture.

Response surfaces were generated for each plate age and media composition combination to predict optimal phytase production based on age of liquid inoculum and duration of SSF. The predicted response surface resulted in maximal phytase production at 1011 U/g substrate with a liquid culture age of 133.79 h from a 14-day plate on M1+ medium and incubated for 226.45 h in SSF. However, for scale-up fermentation studies, we propose using the 7-day plate culture in M1+ medium, resulting in optimal phytase production of 916 U/g substrate at a shorter liquid culture age (85.58 h) and a solid-state cultivation time of 206.54 h. This treatment combination is of interest from an economic point of view. The predicted phytase values at selected operational conditions from the response surface models were within the range verification studies conducted in the laboratory, which confirms the ability of response surface methodology to predict and optimize the fermentation process.

This study has demonstrated the impact of inoculum culturing conditions on fermentation yield and overall performance of the process. Statistically based experimental designs are valuable tools

Table 7 Experimental (verification studies) and predicted results (from the response surface model) of the dependent variable, phytase activity (U/g substrate), of verification fermentation studies under selected operational conditions in SSF systems

Treatments (SSF inoculated with liquid culture from)	Dependent variable	Operational conditions		Experimental value	Predicted value
		Age of liquid culture (h)	Duration of SSF (h)		
7-day plate, M1+ medium	Phytase (U/g substrate)	96	216	1025±100	910.23
14-day plate, M1+ medium	Phytase (U/g substrate)	96	216	1089±110	981.62
7-day plate, M1− medium	Phytase (U/g substrate)	72	192	838±58	827.40
14-day plate, M1− medium	Phytase (U/g substrate)	72	192	897±74	838.19

in optimizing operational conditions and inoculum performance for maximal phytase production. The strong relationship between growth and yield in the fermentation process shows promise for establishing consistency and reliability of the production process.

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