Research Article

Improvement of Phytase Activity by a New *Saccharomyces cerevisiae* Strain Using Statistical Optimization

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Using statistical optimization, we enhanced the activity of phytase by a new *Saccharomyces cerevisiae* strain cultured in mineral medium. Concentrations of carbon source and inducer of phytase production were optimized using a 2^2 full factorial CCD and response surface methodology (RSM). Urea was fixed as nitrogen source in culture medium (0.15%, w/v). The culture medium consisting of 2.5% sucrose and 0.5% sodium phytate optimally supported the maximum phytase activity. In addition, we found that culture of the yeast at 35°C with shaking at 150 rpm supports maximum phytase production. The validity of this model was verified by culturing the organisms in flasks on a shaker. Using the optimized media and growth conditions, we obtained a 10-fold improvement in the production of phytase by *S. cerevisiae*.

1. Introduction

Phytate degradation is an important metabolic process in many biological systems. Although phytate is the major storage form of phosphorus found in cereals, grains, legumes, pollens, and seeds [1], it is not widely available to monogastric animals, such as swine and poultry. Consequently, inorganic phosphorus, a nonrenewable and expensive mineral, is added to the diets of pigs, fish, and poultry to meet their nutritional phosphorus requirements [2, 3]. However, any phosphorus unused by the animals' body is excreted, causing environmental problems in areas of intensive livestock production [3, 4]. In addition, phytic acid acts as an antinutrient because it chelates nutritionally important metals, such as iron, zinc, magnesium, and calcium and binds proteins and lipids, thus diminishing the bioavailability of these important nutrients [5].

Phytase (*myo*inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyzes the hydrolysis of phytate to *myo*-inositol pentakisphosphate and orthophosphate. Improving the digestibility of proteins and increasing the availability of phosphorus and other minerals, which are usually chelated by phytic acid [6], diminishes the antinutritive properties of phytate, and prevents environmental pollution. Thus, the ideal enzyme for the catalysis of such processes should have high catalytic specificity, resistance to proteolysis [7], and stability at elevated temperatures and under acidic conditions [8].

The process of fermentation is significantly influenced by various physical and chemical factors. In addition, phytase production is affected by growth conditions, the strain and substrate used for culture, and the availability of nutrients [2]. Statistical plans are currently used to find ways to enhance phytase production at a reduced cost. The use of response surface methodology (RSM) in biotechnological processes has gained great importance in the optimization of enzyme production because it helps to determine the optimum parameters for enzyme activity [9–16].

Phytase is widely distributed in plants, animal tissues, and microorganisms. Phytases are produced by yeast either naturally [17–20] or by processes based on recombinant DNA technology using different yeast strains [21–26]. *Saccharomyces cerevisiae* possesses several properties that make it useful for use in biotechnological applications, such as its resistance to high sugar and alcohol concentrations [27] and a high growth rate at increasing temperatures [28]. Phytases

have been studied in different yeast strains, such as baker's yeast [29], and an extracellular acid phytase from *S. cerevisiae* was recently purified and characterized [30]. Additionally, a phytase-producing yeast strain, identified as *S. cerevisiae* strain zi (EU188613), was isolated from soil samples from Sao Paulo, Brazil [31]. This strain is potentially a new source of thermostable phytases of commercial interest, particularly because the screen for this yeast was performed using gradual temperature increases. In this study, our aim was to optimize the medium composition and culture conditions to maximize the production of phytase by *S. cerevisiae* strain zi (EU188613) using statistical designs.

2. Material and Methods

2.1. Yeast and Preinoculum Preparation. The yeast strain was isolated from a sample of soil, identified as *S. cerevisiae* strain zi (EU188613) by molecular taxonomy [31] and deposited in the CBMAI (Coleção Brasileira de Microrganismos de Ambiente e Indústria) collection. The yeast cultures were maintained in assay vials containing yeast malt agar (YMA) and stored at 4°C until they were used for preparation of the preinoculum, which was done by suspending the spores in 2.5 mL of sterile water. The cell suspension was used to inoculate the culture medium.

2.2. Phytase Assay. Phytase production was measured using a colorimetric method by following the release of inorganic phosphate from phytic acid. Free inorganic phosphate was assayed in the culture supernatant based on the concentration of phosphate released after hydrolysis of sodium phytate by phytase [32]. For this purpose, 150 μL of enzyme solution were mixed with 600 μL of 0.1 M Tris-HCl (pH 7.0) supplemented with 2 mM sodium phytate and 2 mM of CaCl$_2$, and incubated at 37°C for 30 min. The reaction was then stopped by addition of 750 μL of 5% trichloroacetic acid, after which 1.5 μL of the color reagent were added to generate phosphomolybdate. The concentration of inorganic orthophosphate (Pi) in this mixture was determined colorimetrically by measuring the absorbance of the solution at 700 nm using a Beckman Coulter DU640 Spectrophotometer (Fullerton, CA, USA). The color reagent was prepared fresh by mixing 4 volumes of 1.5% (w/v) ammonium molybdate solution supplemented with 5.5% (v/v) sulfuric acid and 1 volume of 2.7% (w/v) ferrous sulfate solution. The results were compared to a standard curve prepared using K$_2$HPO$_4$ as a source of inorganic phosphate at concentrations ranging from 0.0448 to 2.8706 μM.

Phytase production was also assayed using the method proposed by Stockmann et al. [24] with adaptations for using a synthetic phosphatase substrate. This method is based on the hydrolysis of 4-nitrophenyl phosphate (4-NPP) to phosphate and p-nitrophenol. The substrate (1000 μL of 5 mM 4-NPP disodium salt hexahydrate) was added to a mixture of 500 μL of 0.8 M sodium acetate buffer (pH 5.0) and 500 μL of enzyme solution. The mixture was incubated at 37°C for 10 min, and the reaction was stopped by the addition of 2000 μL of 0.1 N NaOH. The concentration of released p-nitrophenol was determined colorimetrically by measuring the absorbance of the mixture at 410 nm using a Beckman Coulter DU640 spectrophotometer (Fullerton, CA, USA). The results were compared to a standard curve prepared using p-nitrophenol at concentrations ranging from 0.0719 to 7.1891 μM.

One unit (U) of phytase activity was defined as the concentration of inorganic phosphate, in μmol, released per min per mL of enzyme preparation (U/mL) under defined reaction conditions.

2.3. Biomass Determination. To determine the dry weight of the yeast, the cell pellets were washed twice with distilled water and dried in preweighed tubes at 60°C to a constant weight.

2.4. Control Culture Medium and Conditions. Standard culture medium (10 g/L sucrose, 0.5 g/L sodium phytate [purchased from Sigma-Aldrich, *Steinheim*, *Germany*], 3.0 g/L (NH$_4$)$_2$SO$_4$, 0.5 g/L MgSO$_4$·7H$_2$O, 0.5 g/L KCl, 0.001 g/L FeSO$_4$·7H$_2$O, 0.0075 g/L MnSO$_4$·H$_2$O and 0.1 g/L CaCl$_2$) was adjusted to a pH of 4.5 and autoclaved at 121°C and 1 atm for 15 min. Fifteen milliliters of culture medium and 1 mL of yeast preinoculum were mixed in conical flasks of 50 mL, which were incubated in a rotary shaker (TECNAL TE 421, Piracicaba, SP, BR) at 150 rpm and 35°C for 120 h. After allowing the yeast to grow, the flasks were centrifuged at 7100 × g and 10°C for 15 min in a Beckman Coulter Allegra X-22R centrifuge (Fullerton, CA, USA). The culture supernatant was then used directly for the phytase assay, and the pellet was used for biomass determinations after 12, 24, 36, and 48 h for determination of the experimental fermentation time.

2.5. Optimization of the Medium Composition for the Production of Phytase Using RSM. The control culture medium was use and the carbon sources (sucrose) and the inducer of phytase production (sodium phytate) were further optimized in the synthetic medium by RSM and by using central composite design (CCD). One milliliter of yeast preinoculum was used in all the experiments. In this manner, we sought to determine the optimal concentrations of these compounds and to study their interactions. Urea was fixed as nitrogen source at concentration of 0.15% (w/v). The concentrations of these two variables (sodium phytate concentration (A) and sucrose concentration (B)) were optimized using the CCD plan and Statistica software (version 7.0). Each variable in the design was studied at five different levels (Table 1). A 2^2 factorial design, with four axial points and three replicates at the center point and a total of 11 experiments were employed (Table 2). The average maximum phytase activity in *S. cerevisiae* cultured in sodium phytate substrate was measured after 36 h of fermentation. The behavior of the system can be explained by the following quadratic equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB, \quad (1)$$

where Y is the predicted response, β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are squared coefficients,

Table 1: Concentration ranges of the medium components as analyzed by RSM.

Factor	Variables	Range examined	Levels				
			$-\alpha$	-1	0	$+1$	$+\alpha$
A	Sodium phytate (% w/v)	0.15–0.55	0.15	0.2	0.35	0.5	0.55
B	Sucrose (% w/v)	0.5–2.5	0.5	0.8	1.5	2.2	2.5

Table 2: Experimental design and results for the optimization of the medium composition for phytase activity using RSM.

Run n°	Sodium phytate (% w/v)	Sucrose (% w/v)	Phytase activity in sodium phytate medium (U/mL)	
			Experimental	Predicted
1	-1	-1	0.35	0.34
2	$+1$	-1	0.36	0.35
3	-1	$+1$	0.35	0.34
4	$+1$	$+1$	0.51	0.50
5	-1.41	0	0.29	0.29
6	$+1.41$	0	0.40	0.41
7	0	-1.41	0.34	0.35
8	0	$+1.41$	0.45	0.46
9	0	0	0.43	0.43
10	0	0	0.43	0.43
11	0	0	0.43	0.43

Table 3: Analysis of variance and regression analysis for the optimization of the medium composition for phytase activity by *S. cerevisiae* in sodium phytate substrate.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value
Regression	0.037781	4	0.009445	24.22
Residual	0.002339	6	0.00039	
Lack of fit	0.002338	4	0.000585	
Pure error	0.000001	2		
Total	0.04012	10		

Coefficient of determination (R^2) = 0.9417; $F_{4;6;0.05}$ = 4.53.

β_{12} is the interaction coefficient, and A, B, A^2, B^2 and AB are the levels of the independent variables. The corresponding ANOVA values are shown in Table 3.

2.6. Optimization of the Fermentation Conditions for the Production of Phytase. The production of phytase in optimized medium was studied under different culture conditions. One milliliter of yeast preinoculum was used in all the experiments. To determine the optimum temperature (A) and agitation (B) for the production of phytase and to study their interactions, RSM using CCD was applied. The two independent variables (A and B) were studied at five different levels ($-\alpha$, -1, 0, $+1$, $+\alpha$; Table 4), and a total of 11 experiments were performed (Table 5). The average maximum phytase

Table 4: Temperature and agitation ranges as analyzed by RSM.

Factor	Variables	Range examined	Levels				
			$-\alpha$	-1	0	$+1$	$+\alpha$
A	Temperature (°C)	25–45	25	28	35	42	45
B	Agitation (rpm)	70–230	70	93	150	207	230

Table 5: Experimental design and results for the optimization of the fermentation conditions for phytase activity using RSM.

Run n°	Temperature (°C)	Agitation (rpm)	Phytase activity in sodium phytate (U/mL)	
			Experimental	Predicted
1	-1	-1	0.53	0.49
2	$+1$	-1	0.45	0.46
3	-1	$+1$	0.40	0.41
4	$+1$	$+1$	0.37	0.43
5	-1.41	0	0.29	0.32
6	$+1.41$	0	0.36	0.31
7	0	-1.41	0.59	0.61
8	0	$+1.41$	0.58	0.54
9	0	0	0.61	0.61
10	0	0	0.62	0.61
11	0	0	0.61	0.61

activity in *S. cerevisiae* cultured in sodium phytate substrate was measured after 36 h of fermentation. The behavior of the system can be explained by the following quadratic equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB, \quad (2)$$

where Y is the predicted response, β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are squared coefficients, β_{12} is the interaction coefficient, and A, B, A^2, B^2 and AB are the levels of the independent variables.

2.7. Statistical Analysis. The analyses were performed in triplicate, and the results are presented as the mean $\pm$ standard deviation. The Tukey test was used to determine significant differences between the mean values for the different components. P values of ≤ 0.05 were considered statistically significant.

3. Results and Discussion

Prior to optimization, the experimental fermentation time was defined as 36 h. The experiments were carried out at the center point at 12, 24, 36, and 48 h. The results indicated that yeast biomass increases with increased fermentation time. However, the activity of phytase when the yeast was cultured in both substrates increased only up to 36 h (0.55 ± 0.01 U/mL and 0.051 ± 0.004 in sodium phytate and 4-NPP substrates, respectively) and decreased significantly after 48 h.

3.1. Optimization of the Medium Components for the Production of Phytase. Different concentrations of urea were analyzed (0; 0.15 and 0.30% w/v) to determine the best concentration that promotes the production of phytase. Higher concentrations of urea result in decreased phytase activity when *S. cerevisiae* is cultured in sodium phytate substrate. At a concentration of 0.15% urea, the activity of phytase was 0.41 U/mL, while at 0.30% urea we observed an activity of 0.33 U/mL. Urea is the preferred nitrogen sources for the production of phytase by *Aspergillus ficuum* [5] and *Pichia anomala* [13].

The concentrations of sucrose and sodium phytate were further optimized by RSM using CCD, and the data were analyzed using the analysis of variance (ANOVA). Table 1 shows the ranges of the selected variables analyzed for their effects on the production of phytase, and the predicted and observed responses are shown in Table 2. The effects of these variables on the activity of phytase in *S. cerevisiae* cultured in sodium phytate substrate can be predicted by the model:

$$Y = 0.4333 + 0.0406A + 0.0387B - 0.0393A^2 + 0.0355AB, \tag{3}$$

where Y is the activity (U/mL) of phytase in sodium phytate, A is the concentration of sodium phytate, and B is the concentration of sucrose. The corresponding ANOVA values are shown in Table 3. The coefficient of determination (R^2) was 0.9417, which accounts for the 94.17% variability of the model. The R^2-value should be between 0 and 1, and a value ≥ 0.75 indicates aptness of the model [13]. The ANOVA shows a high performance of the F-value for the regression analysis (the calculated values are approximately 5-fold higher than those listed), which indicates that the terms of the model are significant. For the analysis of the activity of phytase in response to sodium phytate, the quadratic parameter for the sucrose concentration was not statistically significant ($P \leq 0.05$) and was added to the lack of fit.

The three-dimensional plot (Figure 1) shows the optimal concentrations and interactions of the sodium phytate and sucrose variables for the production of phytase. At high concentrations of sodium phytate and sucrose (0.55% and 2.5% w/v, respectively), we observed an increase in the production of phytase. The maximum predicted activity for phytase was 0.50 U/mL, while the experimental activity was 0.51 U/mL, indicating that the predicted and experimental values were in agreement.

The statistical model for phytase activity in response to sodium phytate was validated by ANOVA and by repeating the experiments using the optimized conditions. Experimentally, we found the activity of phytase to be 0.49 ± 0.01 U/mL, while the predicted activity was 0.50 U/mL. Based on these results, sucrose at a concentration of 2.5% was used to further optimize the fermentation conditions, and due to its high cost, we used sodium phytate at a concentration of 0.5%.

3.2. Optimization of the Fermentation Conditions for the Production of Phytase. The temperature and agitation conditions were also optimized by RSM using CCD. Table 4 shows
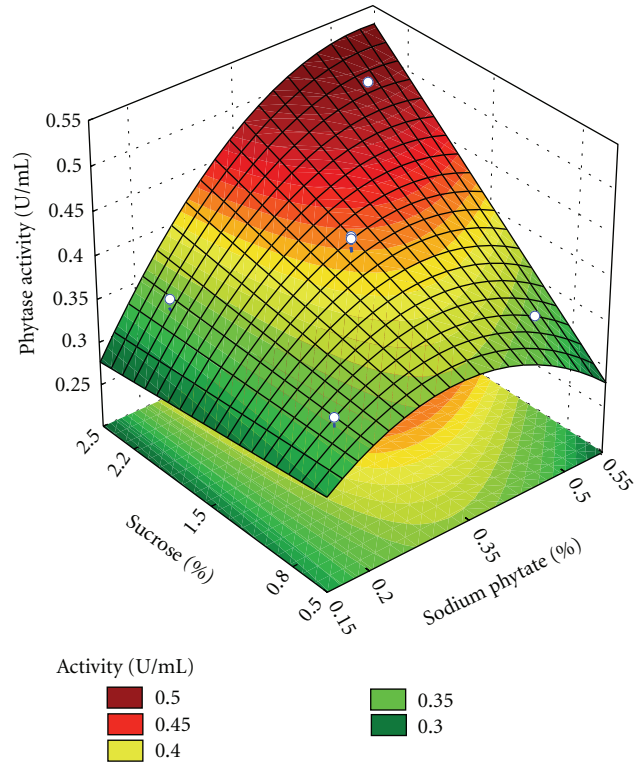

FIGURE 1: Response surface graph showing the effect of the interaction between sodium phytate and sucrose on the activity of phytase.

TABLE 6: Analysis of variance and regression analysis for the optimization of the fermentation conditions for phytase activity by *S. cerevisiae* in sodium phytate substrate.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value
Regression	0.12658	1	0.13162	57.59
Residual	0.01978	9	0.001643	
Lack of fit	0.01972	7	0.002818	
Pure error	0.00006	2	0.00003	
Total	0.14636	10		

Coefficient of determination (R^2) = 0.8648; $F_{1;\ 9;\ 0.05}$ = 5.12.

the different temperature and agitation ranges analyzed for their effects on phytase production, and Table 5 shows the experimental and predicted phytase activity values obtained. The effects of these variables on the activity of phytase in *S. cerevisiae* cultured in sodium phytate substrate can be predicted by the model:

$$Y = 0.6133 - 0.1485A^2, \tag{4}$$

where Y is the activity of phytase (U/mL) in sodium phytate and A is the temperature. This optimized model for the activity of phytase in response to sodium phytate was validated by ANOVA, and the results are shown in Table 6.

The quadratic parameter for the temperature was a significant term in the model for phytase production ($P \leq 0.05$). The R^2 was calculated to be 0.8648, which accounts for
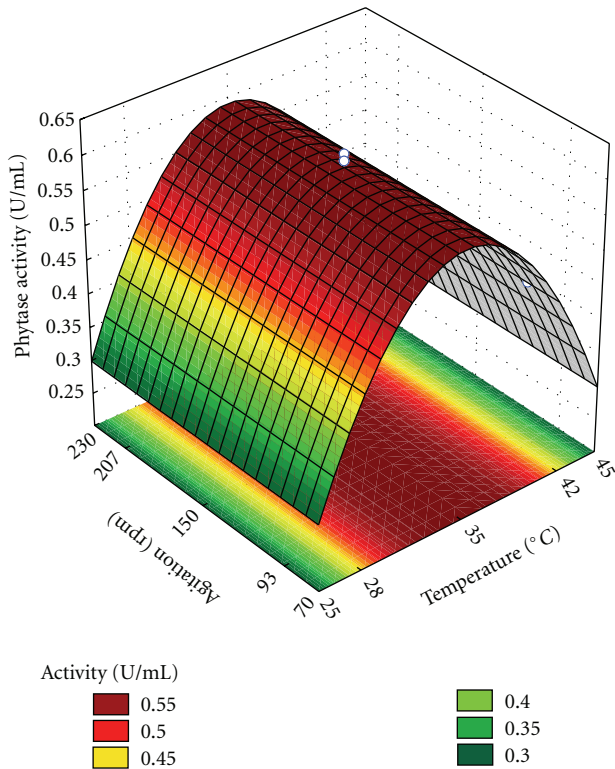
FIGURE 2: Response surface graph showing the effect of the interaction between temperature and agitation on the activity of phytase.
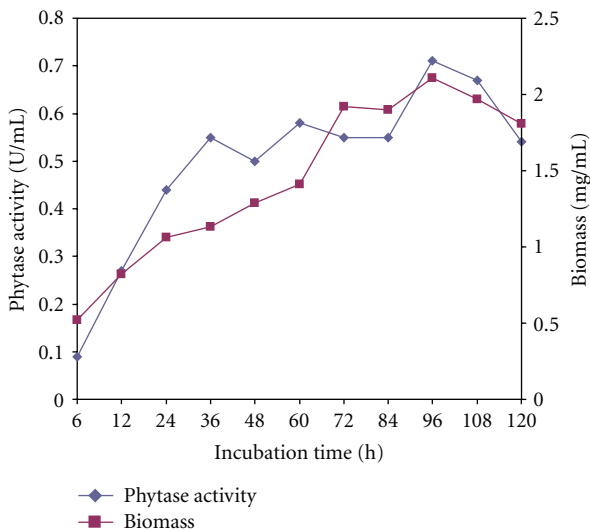

FIGURE 3: Phytase activity and yeast biomass at different time points.

the 86.48% variability of the model. The high F-value for the regression analysis (the calculated values are approximately 11-fold higher than those listed) indicates that the terms of the model are significant.

The three-dimensional plot shows the interaction between temperature and agitation for the production of phytase (Figure 2). The results show that the highest phytase

activity (0.62 U/mL) in response to sodium phytate was obtained at central point conditions (35°C and 150 rpm), which were previously used. The maximum experimental phytase activity obtained was 0.62 U/mL, whereas the predicted value was 0.61 U/mL.

The model was also validated by repeating the experiments using the optimized conditions. The predicted activity of phytase was 0.61 U/mL, while the experimental activity was 0.58 ± 0.02 U/mL.

The phytase activity obtained in unoptimized medium was 0.06 U/mL after 24 h of fermentation by *S. cerevisiae*. After optimization of the medium composition and the fermentation conditions, the activity of phytase in response to sodium phytate was approximately 10-fold higher (0.62 U/mL). After statistical optimization, the activity of phytase increased 1.75-fold in *P. anomala* cultured in synthetic medium [10], 1.7-fold in *Aspergillus ficuum* [11], 1.3-fold in *Rhizomucor pusillus* [12], 3.73-fold in *Sporotrichum thermophile* [15], and 1.8-fold in *M. racemosus* [33].

The activity of phytase in response to sodium phytate after 120 h of fermentation by *S. cerevisiae* was analyzed using the following optimized conditions: 0.5% sodium phytate, 2.5% sucrose, 35°C and 150 rpm. The activity of phytase in response to sodium phytate and the biomass of *S. cerevisiae* at different time points are shown in Figure 3. The activity of phytase and the biomass of the yeast increased progressively over time, and the maximum phytase activity (0.710 ± 0.002 U/mL) and biomass (2.11 ± 0.03 mg/mL) were obtained after 96 h of fermentation. However, the enzyme yield significantly declined after further incubation times ($P \leq 0.05$), which is possibly due to the reduced levels of nutrients available in the medium, as reported by Roospesh et al. [34]. In agreement with this result, the maximum phytase activity and biomass for *M. racemosus* was reported to be obtained after 96 h of fermentation, suggesting a relationship between enzyme production and maximum growth [34].

S. cerevisiae produces extracellular phytase in simple mineral medium, and RSM has proved to be effective for optimizing the activity of phytase. Few studies have reported the use of RSM to improve the production of phytase by yeast, and this is the first study that was carried out to enhance the production of phytase by *S. cerevisiae*. Thus, this yeast strain has potential applications for the reduction of phytate in animal feed.

Acknowledgment

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