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Optimization of Glucoamylase Production by *Aspergillus niger* in Solid-State Fermentation

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Abstract

Glucoamylase production by *Aspergillus niger* in solid-state fermentation was optimized using factorial design and response surface techniques. The variables evaluated were pH and bed thickness in tray, having as response enzyme production and productivity. The bed thickness in tray was the most significant variable for both responses. The highest values for glucoamylase production occurred using pH 4.5 and bed thickness in the inferior limits at 2.0–4.2 cm. For productivity, the optimal conditions were at pH 4.5 as well and bed thickness from 4.4 to 7.5 cm. The optimal conditions for glucoamylase production while obtaining high activity without loss of productivity were pH 4.5 and bed thickness in tray from 4.0 to 4.5 cm, which resulted in an enzyme production of 695 U/g and productivity of 5791 U/h.

Index Entries: *Aspergillus niger*, factorial design; glucoamylase; optimization; solid-state fermentation.

Introduction

Glucoamylase (EC 3.2.1.3.1; 4-α-D-glucan glucohydrolase) is an enzyme that cleaves the glucose units from the nonreducing ends of starch and glycogen. Although a number of glucoamylases from cultures of various microorganisms have been screened for potential industrial applications, commercially available enzyme preparations are at present obtained from either *Aspergillus* sp. or *Rhizopus* sp. *(1)*. Glucoamylases are used mainly in glucose syrup production, in high-fructose corn syrup, and in whole grain and starch hydrolysis for alcohol production *(2)*.

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Solid-state fermentation is generally defined as the growth of microorganisms on solid materials in the absence or near absence of free water. This process is an alternative for enzyme production, especially glucoamylase, because solid-state fermentation has potential advantages over the submerged state of fermentation with respect to simplicity of operation, low energy requirement, and high productivity *(3,4)*.

Tray fermentors are the simplest of all types of fermentors and may be wooden, metallic, or plastic *(5)*. In any fermentation process, the fermentor provides the environment for the growth and activity of the microorganisms, which bring about the biologic reaction *(6)*. Several factors that influence solid-state fermentation include selection of a suitable substrate, initial pH, bed thickness, initial moisture content, and incubation time. Knowledge of the optimum conditions for the fermentation agent's development can promote maximum production of the wanted metabolite *(7)*.

The influence of the fermentation conditions on glucoamylase production has been evaluated and reported in several works. Singh and Soni *(8)* optimized glucoamylase production by studying different substrates, the level and nature of moistening agent, the temperature, the presence or absence of carbon, and nitrogen and mineral supplements. Ellaiah et al. *(9)* investigated some factors that influence glucoamylase production in solid-state fermentation, including the initial pH and moisture content, the incubation time, the level of salt solution, and the effect of various substrates. Bertolin et al. *(4)* investigated the effect of maltose and soluble starch on batch and fed-batch solid-state fermentation for glucoamylase production from *Aspergillus awamori*. Sanzo et al. *(10)* concluded that the type of inoculum had no influence on glucoamylase activity for the semicontinuous solid-state cultivation process of rice bran by *Aspergillus niger* NRRL 3122.

In solid-state fermentation, the amount of substrate is decisive for the development of microorganisms, because a compact cultivation reduces the availability of oxygen. The optimum initial pH of the medium has not yet been exploited for this NRRL 3122 strain for solid-state fermentation. It is fundamental in enzyme production at a large scale to maintain maximum activity without affecting productivity. Therefore, it is important during the optimization process to take productivity into consideration.

Factorial design and response surface techniques are important tools to determine optimal process conditions. Factorial design of a limited set of variables is advantageous compared to the conventional method, which varies a single parameter per trial. The conventional approach frequently fails to locate optimal conditions owing to its failure to clearly show possible effects of interactions between parameters *(11)*.

In this article, we report the production of glucoamylase by *A. niger* NRRL 3122 in solid-state fermentation in trays. The factors pH and bed thickness in tray were evaluated in a full factorial design 2^2 plus axial and central points and response surface analysis, to establish the optimal conditions for glucoamylase production.

Materials and Methods

Inoculum

A. niger NRRL 3122 was maintained at 4°C in a test tube containing potato dextrose agar slant. The inoculum was obtained by incubating *A. niger* in a Roux bottle at 30°C for 7 d. The spores were suspended in sterile water containing 0.2% Tween-80. The suspended spore concentration was estimated by direct microscopy counting using a Neubauer cell-counter chamber.

Composition of Medium

The substrate used was defatted rice bran. For each 100 g of medium, 0.5 g of $CaCO_3$, 4.0 g of K_2SO_4, 7.0 g of $(NH_4)_2HPO_4$, and 3.2 g of urea was added as nutrient and 15 g of rice straw as medium support. Gelatinization of starch and sterilization of medium were done at 121°C for 15 min *(3)*.

Fermentation Conditions

The substrate was inoculated with 4×10^6 spores/g after the resulting medium had 50% moisture content and a pH according to the experiment. To obtain a moisture content of 50%, the volume of the salt solution, the suspension of spores, and the volume used for the adjustment of pH was considered, and if the amount did not reach the moisture desired, distilled water was added. The medium was homogenized and distributed into plastic trays (18×24×9.5 cm) and incubated at 30°C for 96 h. At 12-h intervals, samples were aseptically collected to determine pH, moisture, and glucoamylase production.

Experimental Design

A full factorial design (2^2 plus star configuration) with three replicates at the center point, which means a total of 11 trials, was performed in order to study the effects of pH and bed thickness in tray on glucoamylase production and productivity (Table 1). The distance of the axial points was ±1.41, calculated from Eq. 1 *(12)*:

$$\alpha = (2^n)^{1/4} \tag{1}$$

in which α is the distance of the axial points, and n is the number of independent variables. In the statistical model, the coded settings were defined as follows: X_1 = pH and X_2 = bed thickness. All data were analyzed with the aid of STATISTICA 5.0 from Statsoft (Tulsa, OK).

Glucoamylase Productivity

Glucoamylase productivity (P) was defined as follows:

$$P = \frac{GPm}{ft} \tag{2}$$

Table 1
Coded Values (in Parentheses) and Real Values for Full Factorial Design, Glucoamylase Production (*GP*) (U/g), and Glucoamylase Productivity (*P*) (U/h) During Fermentations

			24 h		48 h		72 h		84 h		96 h	
Run	pH	Bed thickness (cm)	*GP*	*P*	*GP*	*P*	*GP*	*P*	*GP*	*P*	*GP*	*P*
1	3.8 (–1)	2.9 (–1)	40.2	510	483.6	3030	754.5	3143	430.4	1530	427.5	1350
2	5.3 (+1)	2.9 (–1)	151.9	1890	290.7	1830	654.3	2726	463.6	1560	461.7	1440
3	3.8 (–1)	7.1 (+1)	23.7	630	137.5	1960	298.4	2901	601.4	5040	176.2	1260
4	5.3 (+1)	7.1 (+1)	42.7	1260	240.3	3500	320	3111	397.1	3290	395.0	2870
5	3.5 (–1.41)	5.0 (0)	22.3	540	104.4	1320	399.7	3330	238.8	1680	236.7	1500
6	5.6 (+1.41)	5.0 (0)	261.5	6540	390.4	4860	375.4	3128	255.9	1860	342.7	2160
7	4.5 (0)	2.0 (–1.41)	537.9	5600	965.5	5025	650	2256	671.6	1975	720.2	1875
8	4.5 (0)	8.0 (+1.41)	224.9	8460	394.4	7380	500.2	5557	432	4590	542.9	5040
9	4.5 (0)	5.0 (0)	42.3	1020	500.9	6240	690.5	5754	514.5	3660	422.6	2640
10	4.5 (0)	5.0 (0)	22.9	540	639.3	7980	664.6	5538	754.1	5340	635.0	3960
11	4.5 (0)	5.0 (0)	25.9	660	358.3	4500	648.5	5404	419.6	3000	432.7	2700

in which *P* is glucoamylase productivity (U/h); *GP* is glucoamylase production (U/g); *m* is quantity of medium in the tray (g); and *ft* is fermentation time (h).

Extraction of Enzyme

Distilled water was added to fermented samples (in a 1:10 proportion) in Erlenmeyer flasks, and the extraction was done in a shaker at 30°C and 200 rpm for 3 h. The samples were then filtered, and the extract obtained was used as the enzyme source. All extractions were conducted in duplicate.

Enzyme Assay

Glucoamylase production was determined by measuring the reducing sugar released with soluble starch as substrate. The reaction mixture consisted of 4% soluble starch (w/v) in 0.1 *M* acetate buffer, pH 4.5, and 0.5 mL of enzyme source in a total volume of 13 mL. The reaction mixture was incubated at 60°C for 60 min. After incubation, the reaction was stopped by placing in boiling water for 5 min, and the amount of reducing sugar was measured by the dinitrosalicylic acid method *(13)*. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to glucose/min under the assay conditions, and enzyme production was expressed as units per gram of fermented dry matter. All values given are averages of three determinations.

Measurement of pH and Moisture Content

The pH was determined using 1.0 g of fermented material in 10 mL of distilled water, and then the mixture was agitated. After 10 min, the pH was measured in the supernatant using a pH meter *(14)*. The moisture content of the medium was estimated by drying 5 g of the wet sample to a constant weight at 105°C and the dry weight was recorded *(14)*.

Results and Discussion

The coded and real values for pH and bed thickness as well as glucoamylase production (U/g) and productivity (U/h) for each run for the factorial design are presented in Table 1. Statistical analysis was performed with data obtained at 72 h of fermentation, because there was no significant increase in glucoamylase production after this time. Enzyme production at 72 h varied from 298.4 U/g (run 3) to 754.5 U/g (run 1). These results are higher than those found by Ellaiah et al. *(9)*, who achieved maximum glucoamylase production of 247 U/g with *Aspergillus* sp. A3 and using wheat bran as substrate.

An estimate of a main effect of variables on the response is obtained by evaluating the difference in process performance caused by a change from the low (–1) to the high (+1) level of the corresponding factor *(15)*. Process performance was measured by the glucoamylase production and productivity responses. Both the *t*-test and *p* value statistical parameters

Table 2
Regression Coefficients for Glucoamylase Production ([*GP*];U/g) and Productivity ([*P*];U/h) After 72 h of Fermentation

	Regression coefficients		*t* (2)		SE		*p*	
	GP	*P*	*GP*	*P*	*GP*	*P*	*GP*	*P*
Mean	667.5	5575	12.33	54.68	54.10	101.95	<0.001[a]	<0.001[a]
pH								
Linear	–14.2	–62.12	–0.42	–0.98	33.30	62.75	0.687	0.420
Quad	–135.3	–1384	–3.38	–18.37	39.98	75.33	0.019[a]	0.002[a]
Bed depth								
Linear	–126.3	724.39	–3.79	11.54	33.30	62.75	0.012[a]	0.007[a]
Quad	–39.6	–861.10	–0.99	–11.43	39.98	75.33	0.366	0.007[a]
1 by 2	30.4	157.13	0.64	1.77	46.86	88.30	0.544	0.210

[a]Significant factor ($p < 0.05$).

were used to confirm the significance of factors studied (Table 2). The *t* value that measured how large the coefficient is in relationship to its standard error was obtained by dividing each coefficient by the standard error. The *p* value is the chance of getting a larger *t* value (in absolute value) by chance alone. A small *p* value suggested that the coefficient was a large signal in comparison to the noise because it was too large to have arisen by chance alone *(16)*. In this case, $p < 0.05$ suggested significance at the 0.05 level.

The results showed that glucoamylase production was more significantly affected ($p < 0.05$) by bed thickness in tray. The change in the bed thickness from the low (–1) to high (+1) level exhibited a negative influence that resulted in a reduction in glucoamylase production on average of 395 U/g at 72 h of fermentation. This reduction is probably owing to a lesser degree of aeration. Similar conclusions were reported by Singh and Soni *(8)*, who evaluated glucoamylase production from *Aspergillus oryzae* HS-3 in solid-state fermentation using different quantities of substrate in trays.

The effect of initial pH on enzyme production was not statistically significant at a 95% confidence level, indicating the ability of the microorganism to adapt in this pH range (3.8–5.3). In addition, an increase in the pH values for all experiments during the fermentation, as a result of metabolic activities, was observed.

Table 2 presents the regression coefficients for glucoamylase production (*GP*) and productivity (*P*) as a function of pH and bed thickness. To construct a second-order model that can predict glucoamylase production (Eq. 3) and glucoamylase productivity (Eq. 4) as a function of bed thickness and pH, analysis of variance (ANOVA) (Table 3) was used to evaluate the adequacy of the fit. The R^2 value provided a measure of how much of the variability in the observed response values could be explained by the experimental factors and their interaction *(15)*.

Table 3
ANOVA for Glucoamylase Production (*GP*) and Productivity (*P*)
After 72 h of Fermentation[a]

Source of variation	Sum of squares		Degrees of freedom		Mean squares		*F*-test	
	GP	*P*	*GP*	*P*	*GP*	*P*	*GP*	*P*
Regression	218,347	18,106,962	2	3	109,173	603,565	15	12.4
Residual	57,883	3,387,948	8	7	7235	483,992		
Lack of fit	56,985	3,325,566	6	5	9497	665,113		
Pure error	898	62,382	2	2	449	31,191		
Total	276,230	23,846,711	10	10				

[a]*GP*, regression coefficient: $R = 0.90$; $F_{0.95;2;8} = 4.46$; *P*, regression coefficient: $R = 0.87$; $F_{0.95;3;7} = 4.35$.

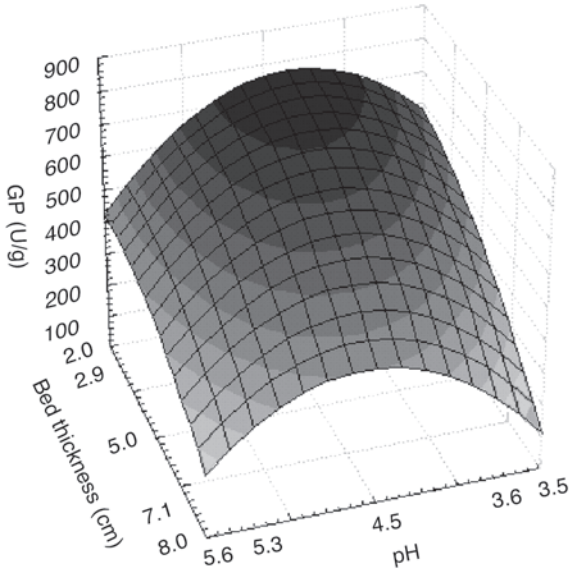

Fig. 1. Response surface for glucoamylase production (*GP*) as function of pH and bed thickness.

Based on the *F*-test, both models are predictive, because calculated *F* values are higher than the critical *F* values. The coded models were used to generate the response surfaces, presented in Figs. 1 and 2.

$$GP = 667.5 - 135.3X_1^2 - 126.3X_2 \quad (3)$$

According to Fig. 1, at the fixed bed depth, high glucoamylase production was recorded at pH 4.5, and at fixed pH a decrease in bed depth led to an increase in glucoamylase production. Therefore, the optimal conditions were at pH 4.5 and bed thickness from 2.0 to 4.2 cm, with an enzyme production above 710 U/g at 72 h of fermentation. These values of optimum

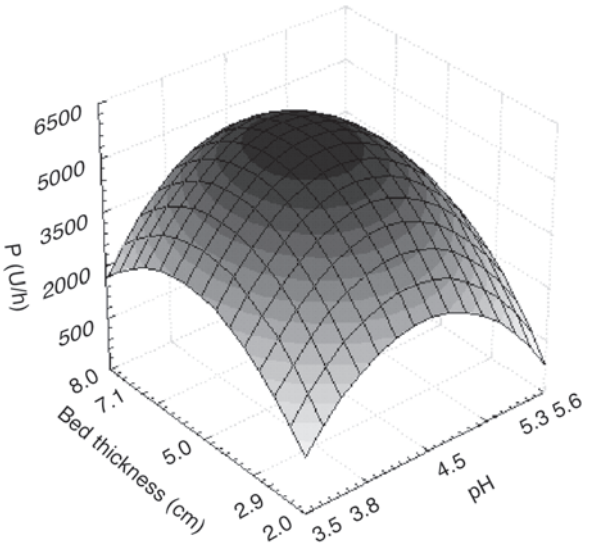

Fig. 2. Response surface for glucoamylase productivity (P) as function of pH and bed thickness.

pH for glucoamylase production by *A. niger* were similar to those previously reported *(17,18)*.

$$P = 5575 - 1384X_1^2 + 724.6X_2 - 861.2X_2^2 \quad (4)$$

Productivity was favored at pH 4.5, the same as for glucoamylase production; however, maximum productivity was obtained using a bed depth in the range of 4.4–7.5 cm. Interestingly, to establish the optimal conditions for both variables evaluated in the experimental design, a pH of 4.5 and a bed thickness from 4.0 to 4.5 cm must be used.

Conclusion

The methodology of full factorial design was shown to be very useful for optimization of the parameters proposed. The variable most relevant to glucoamylase production is the bed thickness, demonstrating that its appropriate study is crucial for enzyme production by solid-state fermentation. The conditions established for glucoamylase production by *A. niger* in solid-state fermentation were pH 4.5 and a bed depth ranging from 4.0 to 4.5 cm. After validation under these conditions, glucoamylase production of 695 U/g and productivity of 5791 U/h were achieved. Thus, glucoamylase production by *A. niger* and the use of defatted rice bran waste is promising for scale-up production.

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