Optimization of Glucoamylase Production by *Humicola grisea* MTCC 352 in Solid State Fermentation

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ABSTRACT

In the industrial processing of starch, the thermostable glucoamylase is employed in saccharification step. The thermophilic fungi Humicola grisea has been used for the glucoamylase production in solid state fermentation. The extracellular glucoamylase is estimated using glucose oxidase – peroxidase assay method. The initial screening studies revealed that wheat bran is the best substrate among the studied agricultural residues. The fermentation parameters were optimized through the response surface approach. By using central composite design, the optimal values of four important parameters viz., mineral salt solution concentration, incubation period, initial moisture content and inoculum size for glucoamylase production were found to be 65 % (v/w), 80 h, 240 % (v/w) and 13 % (v/w) respectively. The experimental activity of 282 U/gds obtained was close to the predicted activity of 288 U/gds. A high R^2 value (0.9741), P values lesser than 0.05 and AARD values (1.98 %) indicate the accuracy of the proposed model.

Keywords: Glucoamylase, *Humicola grisea*, Response surface methodology, Solid state fermentation

INTRODUCTION

Glucoamylase (EC 3.2.1.3) is an endo-acting enzyme which cleaves both α-1,4 and α-1,6 linkages of starch and related polymers, capable of converting it completely into glucose, when incubated for longer periods (James and Lee, 1996). One of the vital process requirements for the industrial application of glucoamylase for starch processing is its use at elevated temperatures. Hence, the thermostable glucoamylase is the tool of choice in an industrial setting (Ramesh and Murty, 2014). Of the various reported glucoamylase producers, the thermophilic fungus, *Humicola grisea*, has been proven to be a steady source of thermostable glucoamylase (Tosi et al., 1993; Campos and Felix, 1995; Ramesh and Murty, 2015).

The conventional method of glucoamylase production employs submerged fermentation (SmF). In the latest industrial practice, glucoamylase production is mainly carried out using solid-state fermentation (SSF) process (Anto et al., 2006). SmF has several disadvantages such as high capital investment, more complex process, less productivity, high water requirement and higher wastewater production, high energy requirement and high cost for downstream processing (Babu and Satyanarayana, 1995; Pandey, 2003).These issues shift the focus towards SSF process for glucoamylase production. There are dual roles played by the solid substrate used in SSF: nutrient supplement and anchorage for the fungal mycelia.

The best suited substrates for enzyme production in SSF are agricultural waste residues (Ellaiah et al., 2002). There are many agricultural waste residues such as sugarcane bagasse, wheat bran, wheat straw, rice straw, rice bran, maize bran, gram bran and oil cakes, that were successfully utilized for glucoamylase production (Ellaiah et al., 2002; Ramachandran et al., 2004; Balkan and Ertan 2007; Bhargav et al., 2008). Apart from agricultural waste residues, cereal flours, waste bread, food wastes, potato residue and tea waste have been effectively used for the production of glucoamylase (Selvakumar et al., 1998; Te Biesebeke et al., 2005; Wang et al., 2009; Lam et al., 2013; Melikoglu et al., 2013). The use of external carbon and nitrogen components along with the solid substrate enhances glucoamylase production (Kunamneni et al, 2005; Prajapati et al, 2013). Mineral salts supplementation is also necessary for glucoamylase production (Bertolin et al., 2003, Bhatti et al., 2007; Negi et al., 2011).

There are many physico-chemical factors influence glucoamylase production in SSF such as the type and concentration of solid substrate used, presence of external carbon and nitrogen source, initial moisture content pH of the medium, the age and size of the inoculum, fermentation temperature and duration of fermentation (Baysal et al, 2003; Ramachandran et al., 2004; Couto and Sanromán, 2006; Balkan and Ertan, 2007). Hence, it is necessary to optimize fermentation parameters after identifying the most suitable substrate to enhance

glucoamylase production. The influence of the fermentation conditions on glucoamylase production has been evaluated and reported in several works. Singh and Soni (2001) 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., (2002) 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., (2003) investigated the effect of maltose and soluble starch on batch and fed-batch solid-state fermentation for glucoamylase production from Aspergillus awamori.

Optimization by the conventional one-variable-at-a-time approach (OVAT) is practiced by keeping all the parameters at a value, while varying a single parameter, at a time. The major disadvantage of OVAT is that it does not include the interaction effects between the variables studied. Also, the net effect of the individual medium constituents on the overall yield is not portrayed. To overcome these disadvantages, the optimization studies can be performed using statistical techniques such as response surface methodology (RSM). Kumar and Satyanarayana, (2004) and Prajapati et al., (2013) successfully applied RSM for the production of glucoamylase. In the current investigation, a response surface approach was used for the optimization of enzyme production by SSF. The process variables optimized were incubation time, moisture level, inoculum size and total mineral salt concentration.

MATERIALS AND METHODS

Materials

Commercial quality rice husk and brans of rice, wheat, black gram and maize were obtained from a rural market in Vellore, India. These were used as solid substrate in SSF. Until a consistent weight was achieved, the substrates were oven-dried at 70°C. Glucose oxidase/peroxidase (GOD-POD) assay kit used was obtained from Agappe Diagnostics Ltd (India).

Microorganism and maintenance

The thermophilic fungus, *Humicola grisea* MTCC 352, was obtained from Microbial Type Culture Collection, Chandigarh, India. The strain was grown on Potato Dextrose Agar (PDA) tubes. The slants were kept at 45°C for a 10-day period. The slants were then stored at 4°C, before use.

Inoculum preparation

The cultivation was initiated with conidial suspension (2 mL), formulated by taking 0.15% Triton X-100 and added to 250 mL conical flasks that contained 100 mL medium (containing 1 g glucose, 200 mg peptone, 50 mg $MgSO_4.7H_2O$, 50 mg $CaCl_2$, 100 mg K_2HPO_4, 200 mg KH_2PO_4 and 500 µL of Vogel's trace elements solution), adjusted to pH 5. A shaking incubator set at 45°C and 100 rpm was used to grow the culture for 4 days.

Enzyme production

Microbial culture using solid substrate was performed in a 250-mL conical flask that contained 5 g of agricultural waste residue and 5-mL mineral salts solution ($MgSO_4.7H_2O$, 2%; KH_2PO_4, 2%). The SSF medium was supplemented with yeast extract and soluble starch (both at, 1% w/w) as external carbon and nitrogen source, respectively. Prior to sterilization, the moisture content in the medium was altered using distilled water. The fermentation process was started by adding 10% inoculum (v/w) as prepared above. To achieve uniformity, the contents of the flask were stirred well before incubation. At stationary conditions, the flasks were incubated for 4 days at 45°C.

Preparation of crude enzyme

When the fermentation period ended, the entire solid medium was subjected to treat with 50 mL distilled water. This was placed in a shaker that was thoroughly agitated at 100 rpm for a 30-minute period. The suspension was then subjected to filtration using filter paper (Whatman grade 1). The permeate was centrifuged for 10 minutes at a speed of 10,000 rpm, leading to the removal of fungal mycelia. The cell-free supernatant was referred to as crude enzyme and was used throughout the experiments.

Glucoamylase assay

A suitable quantity of crude enzyme was allowed to react with 1% (w/v) soluble starch solution in 50 mM citrate buffer (pH 5.5), at 60°C for the duration of 10 min. The total amount of glucose formed was quantified using Glucose oxidase – peroxidase (GOD-POD) assay kit. A unit of glucoamylase activity is expressed as that quantity of glucoamylase which produces 1 µmole of glucose from starch (soluble) per minute under assay settings.

Central composite design (CCD) and Response surface methodology

RSM derived from the CCD of experiments was made used to optimize four significant factors (mineral salt solution concentration, incuba-

tion period, initial moisture content and inoculum size) for glucoamylase production in SSF. Mineral salt solution concentration, incubation period, initial moisture content and inoculum size were respectively symbolized as X_1, X_2, X_3, and X_4 (Table 1). The design contained two factorial points (–1 and +1), two star points (–2 and +2) and a middle point (0) to estimate the variability of the process with glucoamylase yield as the response.

Table 1. Ranges of the independent variables used in central composite design.

Symbol	Variable	Coded level				
		-2	**-1**	**0**	**1**	**2**
X_1	Mineral salt solution concentration (% v/w)	20	40	60	80	100
X_2	Incubation Period (h)	48	72	96	120	144
X_3	Initial moisture content (% v/w)	100	200	300	400	500
X_4	Inoculum size (% v/w)	10	15	20	25	30

A total of 31 experiments were performed according to the matrix, based on a 4-factor, 5-level CCD (Table 2). The results obtained from experiments were built into a quadratic expression, as a function of the four factors with coded values and is given in equation 1.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y denotes dependent variable's predicted response (glucoamylase yield), β_0 denotes constant offset term, β_i denotes linear effect, β_{ij} and β_{ii} denotes quadratic effect and squared term, respectively. X_i and X_j denote coded independent variables for statistical designs as per equation 2.

$$X = \frac{U - U_0}{\Delta U} \quad (2)$$

where X denotes independent variable's coded value, U & U_0 denotes independent variable's real value and real value on center point, respectively. ΔU denotes value of step change.

Statistical analysis

Statistical analysis of the model developed by CCD was analyzed by Analysis of variance (ANOVA) concept, by making use of the statistical software package MINITAB-17.1.0 (MINITAB Inc., PA, USA). The polynomial model was statistically verified by using various parameters like linear regression coefficient R^2, F- value and absolute average relative deviation (AARD).

RESULTS

Screening of agricultural waste residue for glucoamylase production

In the present study, five different substrates, *viz*., rice bran, wheat bran, rice husk, black gram bran & maize bran were tried for extracellular glucoamylase production. Glucoamylase yield of solid state fermentation on various agricultural wastes with 50% of initial moisture is displayed in Figure 1. It was observed that the type of substrates for culturing *Humicola grisea* played a significant role in the production of glucoamylase. Among the five agricultural substrates studied, the maximum glucoamylase yield was obtained with the medium containing wheat bran. On the other hand, lowest enzyme yield was observed with black gram bran. The substrate suitability for glucoamylase production is as per the following order: wheat bran > maize bran > rice bran > rice husk > black gram bran. Thus, wheat bran was selected as the best source for the production of glucoamylase production in the subsequent experiments.

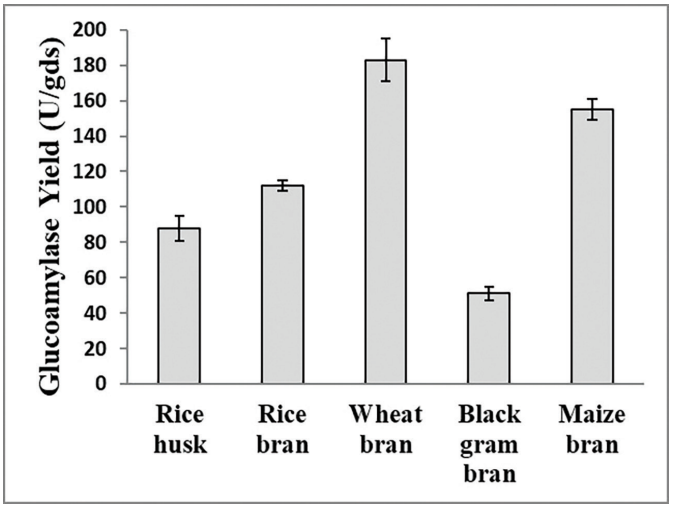

Figure 1. Influence of agricultural waste residues on glucoamylase production.

Optimization of fermentation parameters by response surface approach

The response surface methodology using CCD was employed for the determination of the optimum value of the four important parameters (mineral salt solution concentration, incubation period, initial moisture content and inoculum size) for glucoamylase production. A total of 31 experiments were conducted as per the design matrix and the resulting glucoamylase yield is displayed in Table 2 along with the predicted glucoamylase yield.

Table 2. Experimental and predicted responses of the CCD.

Trial	Coded variable level				Glucoamylase yield (U/gds)	
	X_1	X_2	X_3	X_4	Observed	Predicted
1	-1	1	-1	-1	225	236
2	0	0	0	2	193	201
3	1	-1	1	-1	224	227
4	1	1	1	1	196	192
5	0	0	0	0	266	272
6	0	0	0	0	270	272
7	-1	-1	-1	1	208	211
8	1	-1	-1	1	204	204
9	0	0	2	0	176	184
10	-1	-1	1	1	202	204
11	0	0	0	0	275	272
12	-1	1	1	-1	221	218
13	0	0	0	-2	281	274
14	0	0	-2	0	224	217
15	1	-1	1	1	198	189
16	-1	-1	-1	-1	251	252
17	0	0	0	0	278	272
18	0	0	0	0	277	272
19	0	2	0	0	237	243
20	-1	-1	1	-1	217	218
21	1	-1	-1	-1	261	269
22	0	0	0	0	269	272
23	0	0	0	0	267	272
24	2	0	0	0	185	187
25	1	1	-1	1	190	191

Table 2. Continued.

Trial	Coded variable level				Glucoamylase yield (U/gds)	
	X_1	X_2	X_3	X_4	Observed	Predicted
26	-2	0	0	0	188	186
27	-1	1	1	1	215	209
28	1	1	-1	-1	257	252
29	0	-2	0	0	262	256
30	1	1	1	-1	227	226
31	-1	1	-1	1	206	200

To explain the production of glucoamylase, the second-order regression model equation in terms of coded values was established and expressed in equation 3.

$$Y = 271.71 + 0.25\,X_1 - 3.25\,X_2 - 8.25\,X_3 - 18.33\,X_4 - 21.2\,X_1 * X_1 - 5.45\,X_2 * X_2 - 17.82\,X_3 * X_3 - 8.57\,X_4 * X_4 - 0.37\,X_1 * X_2 + 2\,X_1 * X_3 - 6.13\,X_1 * X_4 + 4\,X_2 * X_3 + 1.12\,X_2 * X_4 + 6.75\,X_3 * X_4 \quad (3)$$

The values of the analysis of variance (ANOVA) for the model are presented in Table 3.

Table 3. ANOVA for the quadratic model.

Variables	Coefficient estimate	Sum of squares	Degrees of freedom	F value	P value
Model	271.71	32,215.8	14	43.04	<0.0001
Linear		9,955.2	4	46.56	<0.0001
X_1	0.25	1.5	1	0.03	0.869
X_2	-3.25	253.5	1	4.74	0.045
X_3	-8.25	1,633.5	1	30.56	<0.0001
X_4	-18.33	8,066.7	1	150.89	<0.0001
Square		20,588.8	4	96.28	<0.0001
$X_{1*}\,X_1$	-21.20	12,851.3	1	240.4	<0.0001
$X_{2*}\,X_2$	-5.45	849.2	1	15.88	0.001
$X_{3*}\,X_3$	-17.82	9,085.1	1	169.95	<0.0001
$X_{4*}\,X_4$	-8.57	2,102.4	1	39.33	<0.0001

Table 3. Continued

Variables	Coefficient estimate	Sum of squares	Degrees of freedom	F value	P value
2-Way Interaction		1,671.8	6	5.21	0.004
$X_{1*} X_2$	-0.37	2.2	1	0.04	0.84
$X_{1*} X_3$	-2.00	64	1	1.2	0.29
$X_{1*} X_4$	-6.13	600.2	1	11.23	0.004
$X_{2*} X_3$	4.00	256	1	4.79	0.044
$X_{2*} X_4$	1.12	20.2	1	0.38	0.547
$X_{3*} X_4$	6.75	729	1	13.64	0.002
Residual		855.3	16		
Lack of Fit		711.9	10	2.98	0.097
Pure Error		143.4	6	43.04	<0.0001
Total		3,3071.1	30	46.56	<0.0001

The model resulted in F-value of 43.04 and coefficient of determination (R^2): 0. 9741. The lack of fit *F* value 2.98. The *P* values were lesser than 0.05 for the linear terms, square effects and interactive effects of mineral salt solution concentration and inoculum size, incubation period and inoculum size as well as initial moisture content and inoculum size. The interaction between the inoculum size and other factors is insignificant for the model obtained. It is clear from the results that the size of inoculum has an independent influence, without interacting with other factors. The *P* value for the lack of fit was found to be 0.097. For the current system, an AARD of 1.98 % was obtained. Figure 2 displays normal distribution of data as a linear trend, which is the indicator that glucoamylase yield obtained from experiments fits the model equation.

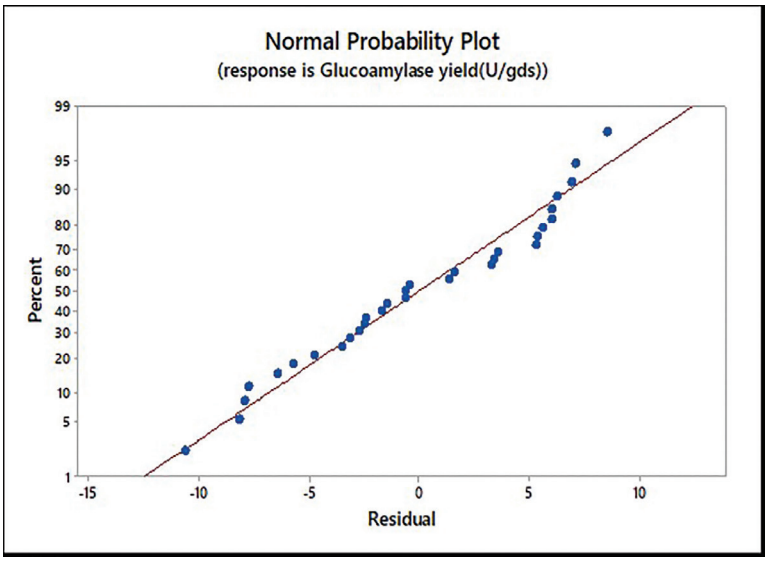

Figure 2. Normal probability plot of glucoamylase production.

In order to visualize the interaction effects between each variable on glucoamylase production, two-dimensional contour plots are shown graphically in Figure 3. The interaction effects between two factors are shown with the other two variables kept constant at their center value. It is clear from the plots that there is a change in glucoamylase production with respect to the low or high levels of the factors. The contour plot between the factors, incubation period & initial moisture content indicates the significant interaction effect and an increase in glucoamylase production at their higher values. The interaction between mineral salt solution concentration & incubation period and mineral salt solution concentration & initial moisture content shows a negative effect (decrease in glucoamylase production at higher values). The same phenomena are numerically shown in Table 3.

The response optimizer tool in MINITAB was used to get a solution for the obtained second-order model equation. The optimum levels of each variable in uncoded units were as follows: mineral salt solution concentration = 65 % (v/w), incubation period = 80 h, initial moisture content = 240 % (v/w) and inoculum size = 13 % (v/w), all of which were located within the experimental range. The predicted glucoamylase yield on wheat bran at the optimum levels of the factors was 288 U/gds. Experiments were performed in triplicates at the optimized values to validate the regression model. Under the optimized conditions, the average of observed experimental values was 282 ± 11 U/gds.

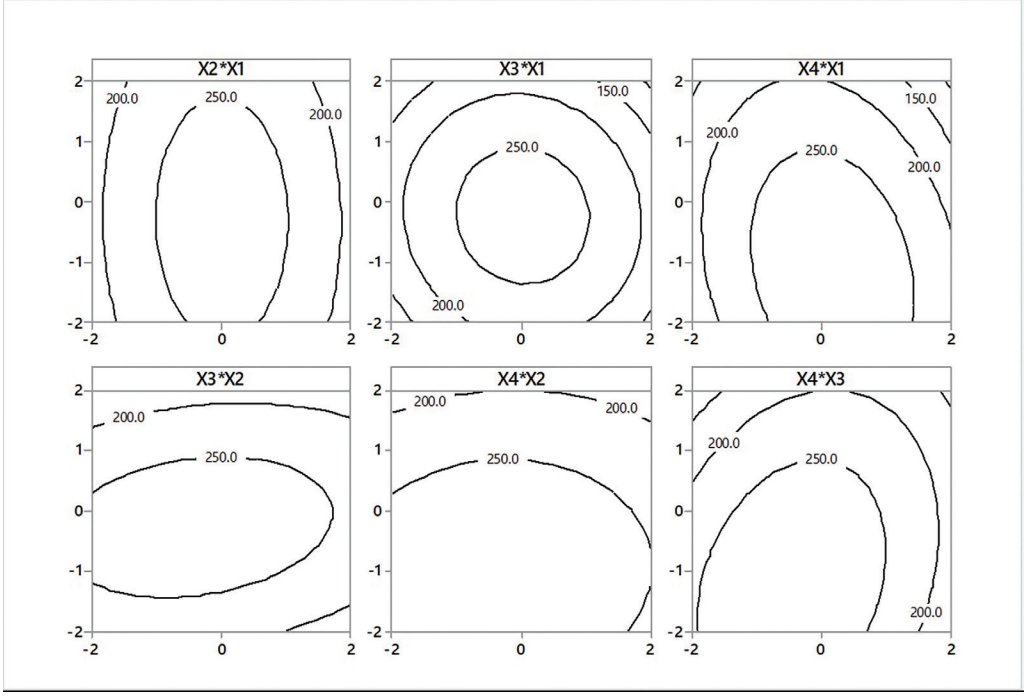

Figure 3. Two-dimensional contour plots for glucoamylase yield (U/gds) (X_1 - Mineral salt solution concentration; X_2 - Incubation period; X_3 - Initial moisture content; X_4 - Inoculum size).

DISCUSSION

The production of extracellular glucoamylase by *Humicola grisea* MTCC 352 was investigated with numerous readily available agricultural waste residues. The screening is aimed towards the selection of better solid substrate, which is a crucial step in the solid state cultivation for the production of desired product. Wheat bran, as the most promising solid substrate for glucoamylase production, has been reported by several researchers (Ellaiah et al., 2002; Anto et al., 2006; Bhatti et al., 2007).

The second-order model obtained from the response surface analysis can be sensibly used, as the difference between the obtained and theoretical yield is meagre. The ANOVA results showed higher model *F* value (43.04) suggests that the second-order model equation obtained was significant. The significance of the second-order model can also be confirmed as results of lack of fit *F* value. The lower *F* value of lack of fit (2.98) compared with higher *F* values of the model suggest the model is significant by means of non-significance lack of fit

(Montgomery, 2005). The significance of the second-order regression model can be similarly established with higher coefficient of determination (R^2): 0. 9741 indicates the extent of correlation between measured glucoamylase yield and model equation. Similarly, the significance of model terms can be established for *P* values lesser than 0.05 (Montgomery, 2005). The linearity of the normal probability plot confirms all major assumptions of the model viz., distribution of errors, same errors of variance, randomization and mean error stand validated. The AARD explains the extent to which the predicted values differ from the experimental values and a lesser value (<5%) is preferred for a good model (Raja and Murty, 2012). For the current model the AARD value of 1.98 % confirms its adequacy.

Contour plots illustrate the substrate to mineral salt solution concentration and initial moisture content have not supported a higher glucoamylase production at their maximum and minimum levels. Lower mineral salt solution concentration causes insufficient nutrient availability, whereas increase in salt concentration was found to inhibit glucoamylase activity (Kunamneni et al., 2005). The resistance for oxygen transport continuously increases with the decrease in porosity of the agricultural residue resulting from an increase in the moisture level of the solid bed. On the other hand, a decrease in moisture content results in lower solubility of nutrients and reduced availability at microbial surface as well as less swelling of the bed (Ellaiah et al., 2002). A similar effect was observed with incubation period. The incubation period for obtaining the maximum glucoamylase yield is decided based on characteristics of the microorganism and is dependent on the product formation rate. The high inoculum size resulted in the high glucose supplementation to the fungus leading to the decrease in glucoamylase yield.

The good correlation between the observed and predicted glucoamylase yield further confirms the adequacy of the model. In addition to this, the optimized glucoamylase yield was found to be higher than the available literature value for the various microorganisms grown on wheat bran medium such as *Aspergillus awamori* [13.7 U/gds] (Bertolin et al., 2003), *Colletotrichum sp.* [61 U/gds] (Prajapati, et al., 2013), *Fusarium solani* [61.35 U/gds] (Bhatti et al., 2007), *Aspergillus awamori* [48 U/gds] (Du et al., 2008) and *Aspergillus sp* [247 U/gds] (Ellaiah et al., 2002).

CONCLUSION

Initial screening study revealed that the type of agricultural waste residue used significantly influences glucoamylase production. Among the tested sources, wheat bran was the best agricultural residue for the glucoamylase production in solid state fermentation. The current study demonstrates the

use of response surface approach for optimization of significant fermentation factors which resulted in enhanced glucoamylase yield. The values of the four parameters were optimized by employing CCD (mineral salt solution concentration: 65 % (v/w), incubation period: 80 h, initial moisture content: 240 % (v/w) and inoculum size: 13 % (v/w)). The proposed second-order model was validated as the difference between the obtained experimental glucoamylase yield of 282 ± 11 U/gds and the predicted glucoamylase yield of 288 U/gds, which was meagre. Thus, the optimized conditions for the solid state fermentation found out in the current study might reduce the overall cost of the production and provides a basis for further studies on a large scale.

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