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Short communication

Inulinase production in a packed bed reactor by solid state fermentation

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ABSTRACT

In this work, production of inulinase was carried out in a packed bed reactor (PBR) under solid state fermentation. *Kluyveromyces marxianus* var. *marxianus* was used to produce the inulinase using pressmud as substrate. The parameters like air flow rate, packing density and particle size were optimized using response surface methodology (RSM) to maximize the inulinase production. The optimum conditions for the maximum inulinase production were: air flow rate – 0.82 L/min, packing density – 40 g/L and particle size – 0.0044 mm (mesh – 14/20). At these optimized conditions, the production of inulinase was found to be 300.5 unit/gram of dry substrate (U/gds).

1. Introduction

Inulinase is an enzyme that hydrolyzes inulin. They target on the β-2,1 linkage of inulin and hydrolyzed it into fructose (Laloux, Cassart, Delcour, Van Beeumen, & Vandenhaute, 1991; Li et al., 2007). Microorganisms are the best sources for production of inulinases because of their easy cultivation and high yields of the enzyme. So far, it has been found that the microorganisms, which can produce high level of inulinases are *Aspergillus* sp., *Penicillium* sp., *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Kluyveromyces* sp., *Cryptococcus* sp., *Pichia* sp., *Candida* sp., etc. (Gao, Chi, Sheng, Ni, & Wang, 2007). It also has been confirmed that yeast strains can produce more inulinase than fungal and bacterial strains (Pandey et al., 1999).

Packed bed bioreactors (PBRs) are widely used in solid state fermentation (Cavalcanti, Gutarra, Freire, Castilho, & Sant'Anna Junior, 2005; Mazutti et al., 2010). The solid medium is retained in the reactor by a static support and air is passed through the bed, ensuring oxygen supply and heat removal. In PBR, the process parameters may be better controlled than in the traditional tray type fermentors. In this work, the linear and interactive effect of process variables like air flow rate, packing density and particle size on inulinase production in a PBR using pressmud as substrate was investigated. The optimum conditions of the process variables that yield maximum inulinase were also found.

2. Materials and methods

Yeast strain *Kluyveromyces marxianus* var. *marxianus* (MTCC-188) was obtained from the Microbial Type Culture collection Centre (MTCC), Chandigarh, India. The strain was maintained on solid medium at 5 °C. The medium composition (g/L) was comprised off the following: yeast extract 3.0; peptone 10.0; dextrose 20.0; and agar 15.0. Cells were harvested from slants and used to inoculate liquid medium.

Pressmud was used as substrate for inulinase production. Fermentation was carried out in a bench scale PBR. The reactor was made up of acrylic column with an internal diameter of 2.5 cm and height of 25 cm. Sterile and moist air was injected at the bottom of the reactor and air flow rate was controlled using a rotameter. The cultivation medium was supplemented with nutrients and carbon source (Dilipkumar, Rajasimman, & Rajamohan, 2010). The medium and PBR were separately sterilized in autoclave. Before packing the solid medium in the sterile reactor, it was inoculated with microorganism and its moisture content was adjusted to 60% (Dilipkumar, 2011). The effect of air flow rate was studied by varying the air flow rates (0.4, 0.8 and 1.2 L/min). After fermentation, the fermented solids were removed from the reactor and transferred to a glass flask for the extraction of inulinase. The effect of

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Table 1
Range and levels of process variables in packed bed reactor.

Process variables	Code	Levels		
		−1	0	+1
Air flow rate (L/min)	A	0.4	0.8	1.2
Packing density (g/L)	B	30	40	50
Particle size (mesh)	C	14/20	10/14	8/10

Table 2
Experimental design for inulinase production in a packed bed reactor.

Run no.	A	B	C	Inulinase activity (U/gds)	
				Experimental	Predicted
1.	−1	1	0	181.0	181.55
2.	0	1	1	175.0	175.71
3.	1	1	0	164.2	161.82
4.	−1	0	1	186.0	184.74
5.	0	0	0	292.6	292.34
6.	−1	−1	0	196.0	198.37
7.	0	−1	−1	265.0	264.29
8.	0	0	0	292.4	292.34
9.	0	−1	1	203.1	201.99
10.	1	0	1	92.0	93.66
11.	0	1	−1	264.0	265.11
12.	1	0	−1	235.5	236.76
13.	0	0	0	292.5	292.34
14.	−1	0	−1	195.0	193.34
15.	1	−1	0	171.0	170.45
16.	0	0	0	292.1	292.34
17.	0	0	0	292.1	292.34

packing density was also studied in the range 30, 40 and 50 g/L. Packing density was varied by adding inert particles made of polypropylene.

RSM was employed to optimize the process parameters to enhance the inulinase production in a packed bed bioreactor. The three independent variables were studied at three different levels (Table 1) and a set of 17 experiments were carried out (Table 2). The statistical software package 'Design Expert 7.1.0' was used to analyze the experimental data. All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated were listed in Table 2. Upon completion of experiments, the average maximum inulinase were taken as the response (Y). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. A second order polynomial equation is:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1, i<j}^{k-1} \sum_{j=2}^{k} \beta_{ji} X_i X_j \quad (1)$$

where Y is the measured response, β_0 is the intercept term, β_i are linear coefficients, β_{ii} are quadratic coefficient, β_{ij} are interaction coefficient and X_i and X_j are coded independent variables. The optimal concentrations of the critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA). The statistical model was validated with respect to inulinase production under the conditions predicted by the model in PBR.

Enzymes were assayed by measuring the concentration of reducing sugars released from inulin or sucrose. The reaction mixture containing 1 ml of diluted crude enzyme and 4 ml of 2% sucrose (dissolved in 0.1 M acetate buffer, pH 5.0) was incubated at 50 °C. After incubating for 30 min, aliquots of 0.5 ml were withdrawn and increase in reducing sugar was estimated by a 3,5-dinitrosalicylic acid method (Miller, 1959) using calibration curve obtained with a standard solution of fructose (Uzunova, Vassileva, Ivanova, Spasova, & Tonkova, 2002). Absorbance was read at 575 nm in Bio spectrophotometer (BL 200 – ELICO, India). A higher absorbance indicated a high level of reducing sugar produced and consequently, a high enzyme activity.

Table 3
ANOVA for inulinase production in PBR.

Source	Coefficient factor	Sum of squares	DF	F	$P > F$
Model	292.34	58,285.65	9	1865.22	<0.0001
A	−11.91	1135.26	1	326.97	<0.0001
B	−6.36	323.85	1	93.27	<0.0001
C	−37.93	11,506.45	1	3314.00	<0.0001
A*B	2.05	16.81	1	4.84	0.0637
A*C	−33.63	4522.56	1	1302.55	<0.0001
B*C	−6.78	183.60	1	52.88	0.0002
A*A	−81.97	28,290.87	1	8148.12	<0.0001
B*B	−32.32	4398.24	1	1266.75	<0.0001
C*C	−33.25	4653.60	1	1340.30	<0.0001

Std. Dev. – 1.86; R^2 – 0.9996; Mean – 222.91; Adj R^2 – 0.9990; C.V. % – 0.84; Pred R^2 – 0.9934; Adeq Precision – 139.02.

3. Results and discussion

SSF were carried out in a PBR for the optimization of critical factors, like airflow rate, packing density and particle size. Experiments were performed according to the Box–Behnken experimental design given in Table 2. Table 3 shows the analysis of variance (ANOVA) for the inulinase production using *K. marxianus* var. *marxianus*. A Model F-value of 1865.22 implies that the model was significant. The Fisher F-test with a very low probability value ($P_{model} > F = 0.0001$) demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). The coefficient of determination (R^2) was calculated to be 0.9996. This implies that more than 99% of experimental data was compatible with the data predicted by the model (Table 2) and only less than 1% of the total variations were not explained by the model. The R^2 value was always between 0 and 1, and a value >0.90 indicates aptness of the model. For a good statistical model, R^2 value should be close to 1.0. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the Adj R^2 0.9990 was also high to advocate for a high significance of the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2. Here in this case the adjusted R^2 value was lesser than the R^2. The Pred R^2 0.9934 was in reasonable agreement with the Adj R^2. The value of coefficient of variation (CV) was also low as 0.84, indicate that the deviations between experimental and predicted values were low. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this work the ratio was found to be 139.020, which indicates an adequate signal.

The experimental results were analyzed through RSM to obtain an empirical model for the response. The results of theoretically predicted response were shown in Table 2. The mathematical expression relating the response with variables was:

$$\text{Inulinase production, U/gds} = 292.34 - 11.91A - 6.36B - 37.93C + 2.05AB - 33.63AC - 6.78BC - 81.97A^2 - 32.32B^2 - 33.25C^2 \quad (2)$$

where A, B and C are the coded values of the test variables, air flow rate (L/min), packing density (g/L) and particle size (mm) respectively.

The significance of each coefficient was determined by Student's t-test and p-values, and was listed in Table 3. The larger the magnitude of the t-value and smaller the p-value, the more significant

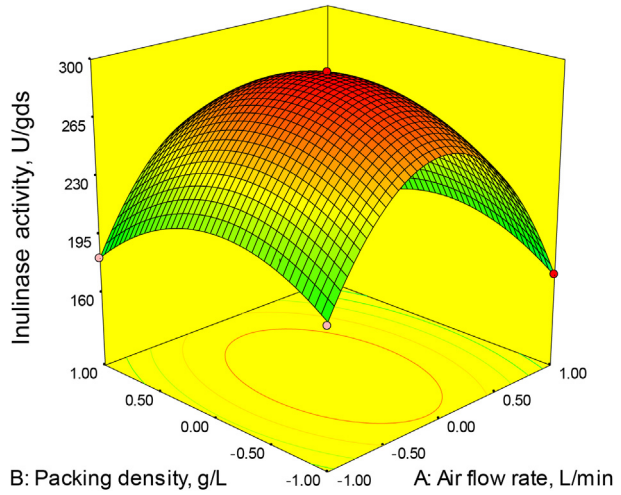

Fig. 1. 3D plot showing the effect of air flow rate and packing density on inulinase production by *K. marxianus* var. *marxianus* using Pressmud in a PBR.

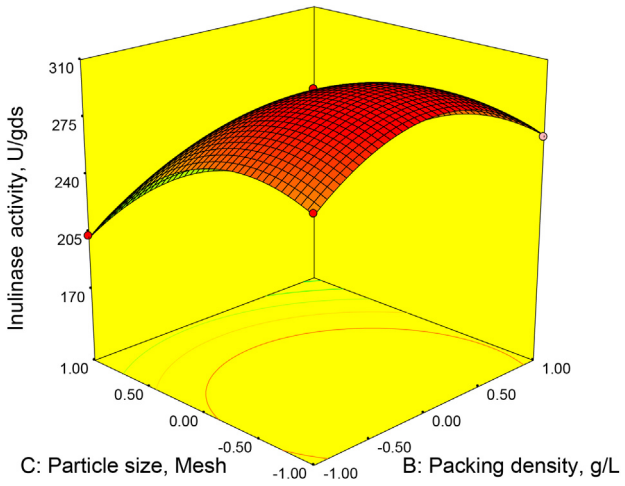

Fig. 3. 3D plot showing the effect of packing density and particle size on inulinase production by *K. marxianus* var. *marxianus* using pressmud in a PBR.

was the corresponding coefficient. Values of "Prob > *F*" less than 0.05 indicate model terms were significant. In this case, *A*, *B*, *C*, AC, BC A^2, B^2 and C^2 were significant model terms for inulinase production. Values greater than 0.05 indicate the model terms were not significant. This implies that the linear and square effects of air flow rate, packing density and particle size were more significant than the other factors. Also interactive effects of *A*–*C*, and *B*–*C* were more significant factors.

The response surface and contour plots were generated for different interactions of any two independent variables, while holding the value of the other variables as constant. Such three-dimensional surfaces give accurate geometrical representation and provide useful information about the behavior of the system within the experimental design. The response surface curves for inulinase production by *K. marxianus* using pressmud were shown in Figs. 1–3. Fig. 1 shows the effect of air flow rate and packing density on inulinase production. From the figure it was observed that inulinase production increases upto 0.82 L/min and after that it decreases. This is because, increase in the aeration rate causes a decrease in the temperature of the culture medium and, consequently, possible enzymes denaturalization can be reduced. But at higher flow rates, the moisture content of the solid substrate was extremely reduced and, therefore, enzymes production was reduced.

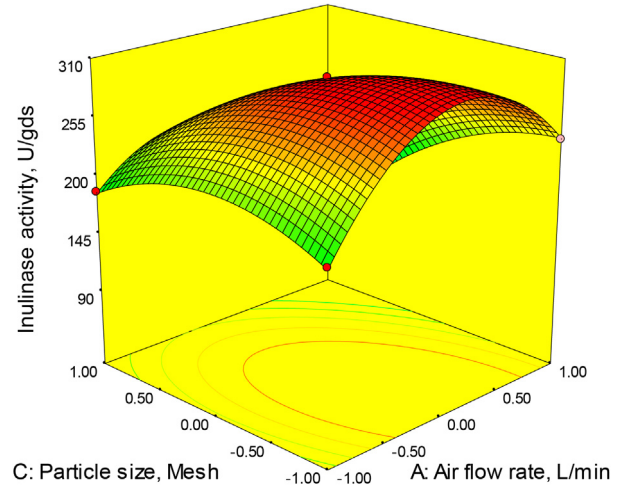

Fig. 2. 3D plot showing the effect of air flow rate and particle size on inulinase production by *K. marxianus* var. *marxianus* using pressmud in a PBR.

Similar results were obtained by Milagres, Santos, Piovan, & Roberto (2004). They found effect of the air flow rate on xylanase production using a PBR. The highest enzyme activity was attained with an intermediate flow rate, however with higher aeration the activity decreased.

Similarly, high packing density restricted the airflow and inhibited the yeast growth. A minimum aeration was required so that the metabolic activity of the yeast was not affected. The optimum value for packing density was 40 g/L. Comparable results were obtained for protein enrichment of lignocellulosic substrate and citric acid production from apple pomace in multi layer PBR (Shojaosadati & Babaeipour, 2002; Shojaosadati, Faraidouni, Madadi, & Mohamadpour, 1999). The increase in particle size decreases the inulinase production. This can be explained by the fact that the substrate acts both as nutrient source and support for microbial growth. Higher particles render mass transfer difficult, due to the decrease in superficial area, and then in the availability of nutrients (Mazutti, Ceni, Di Luccio, & Treichel, 2007). This is clearly depicted in Figs. 2 and 3.

Optimum conditions for the maximum inulinase production were determined. Second order polynomial models obtained in this study were utilized for each response in order to determine the specified optimum conditions. The sequential quadratic programming in MATLAB 7 was used to solve the second-degree polynomial regression equation. The optimum values obtained by substituting the respective coded values of variables are: air flow rate – 0.82 L/min, packing density – 40 g/L and particle size – 0.0044 mm (mesh – 14/20). At these optimized conditions, experiments were carried out in the packed bed reactor. A maximum inulinase production of 300.5 U/gds was achieved at the optimized condition. In Table 2, Run no.5, a maximum inulinase activity of 292.6 U/gds was obtained and it was increased to 300.5 U/gds at the optimized condition using RSM.

4. Conclusions

In this study, PBR was successfully employed for the production of inulinase by *Kluyveromyces marxianus* var. *marxianus*. The process parameters viz. air flow rate, packing density and particle size were optimized using RSM. At the optimized condition a maximum inulinase production of 300.5 U/gds was achieved. This is higher than the un-optimized condition. The results show a close concordance between the experimental and predicted values obtained

by RSM. Hence the RSM can be effectively used to enhance the production of inulinase in PBR.

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