Optimization of process parameters by RSM in a packed bed reactor on production of inulinase using *streptomyces species* from copra waste

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Abstract
In this research work, inulinase produced from coprawaste used as a substrate in a reactor having packed bed (PBR) by solid state fermentation using Streptomyces sp. used for synthesis of inulinase was studied. Air flow rate, initial moisture content, packing density and particle size were optimized by response surface methodology (RSM).

The optimum conditions to obtain maximum production of inulinase were: air flow rate - 0.78 liter /minute,Initial moisture content-53%, packing density - 45 grams/ liter and particle size - 8/10 mesh. In these optimized conditions, maximum inulinase activity was discovered 176.4 unit/gram of dry substrate (U/gds).

Keywords: *Streptomyces sp.*, RSM, Packed bed reactor, Coprawaste.

Introduction

Inulinases are enzymes of industrial significance that function the β-2,1linkages in inulin. Depending on their catalytic activity on the substrate, they are included in both the transferase and hydrolases classes. Inulinases separate β-2, 1 fructofuranosidic inulin bonds by either exo-or endo-action. Exo-inulinase (EC 3.2.1.80; β–2–1–D-fructanfructohydrolase) works sequentially on β-2,1 inulin bonds to create fructose while endo-inulinase (EC 3.2.1.7; β-2-1-D-fructanfructan hydrolase) works randomly to release fructooligosaccharides. The two key applications of inulinases are the development of high fructose syrup and fructooligosaccharides[1,2].

Other applications of inulinases include bioethanol processing, single-cell oil, lactic acid, citric acid, pullulan etc.[3] Fructose is an artificial sweetener with low calories that has many technological advantages over traditional sweeteners such as sucrose, while fructo-oligosaccharides are strong prebiotics.

Most fructose is conventionally manufactured from starch via a multi-enzymatic cycle involving α-amylase, amylo-glucosidase and glucose isomerase. This process yields approximately 45 percent of fructose content.

Specific techniques such as ion exchange have also been developed to give D-fructose syrups over 90%, but these techniques increase production costs. An alternative and attractive process is the enzymatic hydrolysis of inulinase, which yields up to 95 percent of the fructose content[4].

The disposal of agro-waste causes increasing problems as most of them are very susceptible to microbial spoilage, thereby restricting further exploitation. On the other hand, the costs of processing, storage and shipping of by-products are important economically limiting factors. Many agro-wastes are also used as feed or as fertilizer. The problem of disposing is compounded further by regulatory problems and restrictions. Consequently, the realistic, effective and environmentally sound use of waste materials from agriculture is becoming more important, particularly because productivity and jobs can suffer. In recent years there has been an increasing interest in optimizing the use of agricultural by-products for the various microbial enzyme production.

Packed bed bioreactor (PBR) is commonly used for fermentation in solid state[5,6]. A static support keeps the solid medium in the reactor and air passes through the bed, ensuring oxygen supply and heat removal. In PBR, the process parameters can be better managed than in conventional fermenter style trays. In this study, the linear and interactive impact of process variables such as air flow rate, initial moisture content, packaging density and particle size on inulinase output in a PBR using copra waste as a substrate have been investigated. The main aim of this study is to address the agricultural waste that was disposed of or used excessively for non-food applications[7].

Material and Methods

Actinomycete strain: *Streptomyces sp.* MTCC-3119 was taken from a stock of the Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. The strain was held at 5 ° C on solid medium. The medium composition consisted of the following: yeast extract 4.0 gram; malt extract 10.0 gram;glucose 4.0 gram; agar 20.0 gram; distilled water 1.0 liter. Its pH was7.2–7.4. Cells were harvested from slants and inoculated with liquid media.

Solid state fermentation: Copra waste is the substrates and it was obtained from the oil mill after mechanical extraction of coconut oil, Chidambaram, Tami Nadu state, India. It was 48 hours sundried and then used as substrate in this research work. In a bench scale PBR fermentation was carried out according to central composite design.

Packed bed reactor: A bench size packed bed bioreactor made of acrylic board had a 4 cm diameter cylindrical shape

and was used 60 cm in height. At operating temperature, sterile and moist air enters at the bottom of the PBR and passes through the bed, leaving the top outlet of the vessel. Flow rate of air was measured and controlled by a rotameter. In an autoclave the substrate and reactor are sterilized separately.

The solid medium was inoculated with the microbes before packing in the sterile reactor and its process parameters are modified based on the central composite design (CCD). The reactor was then packed with sun-dried substrates, supplemented with the nutrients picked under their optimum conditions[8].

The experiments were conducted for five days during the preliminary screening cycle and it was found that the highest output of inulinase was obtained at 24 hours. Therefore, all the tests were performed for 24 hours. The range and level of process variables were given in table 1.

Central composite design (Table 2) has been performed to optimize the inlet air flow rate, initial moisture content, packing density and particle size. Density of the packaging was varied by the introduction of inert polypropylene particles. RSM was used to optimize process variables to increase inulinase performance in a packed bed bioreactor.

The four process variables were independently analysed at five different levels shown in table 1 and table 2 displaying a total of 30 experiments. The Design Expert software (7.1.5) was used to analyze the experimental data. The fermented solids are removed from the reactor after 24 hours of fermentation and transferred to an Erlenmeyer flask and the inulinase was extracted.

Extraction of inulinase: After fermentation, 5 volumes of distilled water were applied to the fermented material contained in the Erlenmeyer flask. The content was agitated on a rotary shaker at 200 rpm for half an hour (at 28^{o}C). The sample was then centrifuged at 15,000 rpm for 20 min and the supernatants analyzed using DNS method[9].

Enzyme assay: Enzymes were analysed by calculating the concentration of reduced sugars released from inulin or sucrose. The mixture of reactions containing 1 ml of diluted crude enzyme and 4 ml of 2 percent sucrose (dissolved in 0.1 M acetate solution, pH 5.0) was incubated at 50 ^{o}C. After incubating for 30 min, aliquots of 0.5 ml were collected and an increase in sugar reduction was measured using a test of 3.5-dinitrosalicylic acid[9] using a typical fructose solution calibration curve.[10]

A 575 nm absorbance in Bio spectrophotometer was read. Higher absorption indicates a high degree of reducing sugar and, therefore, a high activity of the enzyme. One unit of inulinase activity (U) was specified as the enzyme amount, which forms 1µmol fructose per minute. Results of the inulinase activity were seen in the units of activity/gram of dry substrate(U/gds.).

Results and Discussion

Experimental methods by response surface methodology (RSM): RSM consists of a collection of analytical techniques used to test the relationship between the cluster of controlled experimental variables and the response measured. A prior understanding of the related bioprocesses is needed for a practical modeling approach. To determine which variables affect *Streptomyces sp.* development of inulinase significantly, triplicate experiments were performed and average inulinase activity was recorded. A second order polynomial equation is used which relates the measured response to the independent variables.

The Design Expert statistic software program was used to evaluate the experimental data. All variables were taken at zero central coded value. Table 1 listed the minimum and maximum ranges of variables under analysis. After the experiments were completed, the average maximum inulinase was taken as the response (Y). A multiple regression analysis of the data was performed to obtain an empirical model that relates the calculated response to independent variables. A polynomial equation in second order is:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

where Y is the measured response, β_0 is the intercept term, βi are linear coefficients, βii are quadratic coefficient, βij are interaction coefficient and Xi and Xj are coded independent variables. By examining contour plots, the optimal concentration values of the critical variables were obtained. The model's statistical analysis was expressed in the form of Analysis of Variance (ANOVA). The statistical model was validated in respect of inulinase output under the conditions predicted by the PBR model.

SSF was performed in a PBR to optimize critical factors including air flow rate, initial moisture content, packing density and particle size. Experiments were carried out in accordance with the central composite design as shown in table 2.

Table 3 describes the Analysis of Variance (ANOVA) for the development of inulinase using *Streptomyces sp*. A model F-value of 30.34 indicates that themodel was relevant. The Fisher F-test with a very low probability value (Pmodel > F = 0.0001) indicates very high significance for the regression model. Goodness of fit, model was tested by coefficient of determination (R^2). The determination coefficient (R^2) was estimated as being 0.9659.

This means that more than 96% of experimental results were consistent with the model's expected results (Table 2) and that the model did not clarify just less than 4% of the total

variations. The value R^2 was always between 0 and 1 and a value > 0.90 suggests the model's suitability. R^2 value should be about 1.0 for a good statistical model. The adjusted R^2 value corrects the R^2 value with respect to the sample size and the number of terms in the model.

The Adj value R^2 0.9341 was also strong to advocate for the model's strong significance. If the model includes several terms and the sample size is not very high, the modified R^2 can be significantly smaller than the R^2. Here the adjusted R^2 value was lower than the R^2 value. The Pred R^2 (0.8035) had fair agreement with the Adj R^2. The coefficient of variation (CV) value was also low as 5.04, indicating that the differences between predicted and experimental values were low. Adequate accuracy measures the ratio of signal to noise. For this work the ratio was 16.549, indicating a sufficient signal. The results of the experiments have been analysed by RSM to obtain an empirical response model.

Table 1
Process parameters range and levels of in packed bed reactor.

Variables in process	Code	Levels				
		-2	**-1**	**0**	**1**	**2**
Air flow rate (litres /min)	A	0.2	0.4	0.6	0.8	1.2
Packing density (grams / litre)	B	20	30	40	50	60
Particle size (mesh)	C	14/16	12/14	10/12	8/10	6/8
Initial moisture content (%)	D	40	50	60	70	80

Table 2
Experimental design table to develop inulinase in a packed bed reactor

Run no.	A: air flow rate (liters/min)	B: packing density (grams/liter)	C: particle size (mesh)	D:Initial moisture content (%)	InulinaseActivity (U/gds)	
					Experimental	**Predicted**
1	0	0	0	-2	110.40	116.763
2	1	1	-1	1	161.51	163.924
3	-1	-1	1	-1	153.24	145.614
4	0	0	2	0	95.00	100.763
5	-1	1	1	1	100.10	100.734
6	0	0	0	0	165.40	165.403
7	0	0	0	0	165.42	165.403
8	2	0	0	0	105.84	110.428
9	1	1	1	-1	114.87	109.859
10	0	-2	0	0	110.30	117.383
11	0	0	-2	0	113.84	120.008
12	1	-1	-1	-1	95.40	89.554
13	-1	1	-1	1	140.20	133.163
14	-1	-1	1	1	98.20	91.893
15	0	0	0	0	165.40	165.403
16	1	-1	-1	1	128.36	121.448
17	-1	1	1	-1	123.92	124.113
18	0	0	0	2	119.71	125.278
19	-2	0	0	0	115.20	122.543
20	1	1	-1	-1	102.10	101.688
21	1	-1	1	1	99.54	100.309
22	0	2	0	0	133.51	138.358
23	-1	1	-1	-1	128.20	122.219
24	0	0	0	0	165.40	165.403
25	-1	-1	-1	1	119.51	119.309
26	0	0	0	0	165.40	165.403
27	-1	-1	-1	-1	139.00	138.708
28	0	0	0	0	165.40	165.403
29	1	1	1	1	144.20	137.773
30	1	-1	1	-1	102.42	102.738

Table 3
ANOVA for production of inulinase with PBR

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
Model	17846.63	14	1274.76	30.34	< 0.0001
A-Air flow rate (litres/min)	108.76	1	108.76	2.59	0.1285
B-Packing density(grams/liter)	220.16	1	220.16	5.24	0.0370
C-particle size(mesh)	659.93	1	659.93	15.71	0.0012
D-Initial moisture content(%)	555.56	1	555.56	13.22	0.0024
AB	2630.92	1	2630.92	62.62	< 0.0001
AC	920.67	1	920.67	21.91	0.0003
AD	1178.03	1	1178.03	28.04	< 0.0001
BC	819.25	1	819.25	19.50	0.0005
BD	39.41	1	39.41	0.94	0.3482
CD	25.13	1	25.13	0.60	0.4514
A^2	3376.87	1	3376.87	80.37	< 0.0001
B^2	4102.22	1	4102.22	97.64	< 0.0001
C^2	2414.95	1	2414.95	57.48	< 0.0001
D^2	5189.09	1	5189.09	123.51	< 0.0001
Residual	630.23	15	42.02		
Lack of Fit	630.23	10	63.02	9.453E+005	< 0.0001
Pure Error	3.333E-004	5	6.667E-005		
Cor Total	18476.85	29			

Std. Dev. 6.48; R^2- 0.9659; Mean 128.23; Adj R^2- 0.9341; C.V. % 5.05; Pred R^2-0.8035; Adeq.Precision-16.549

Table 2 showed results of the theoretically predicted response. The mathematical expression relating the variables to the response was:

Inulinase activity (U/gds) =+165.40+2.13 * A-3.03* B+5.24 * C -4.81 * D+12.82 * A * B +7.59 * A * C-8.58 * A * D+7.16 * B * C +1.57 * B * D – 1.25 * C * D-11.10 * A^2-12.23 * B^2-9.38 * C^2-13.75 * D^2

where A, B, C and D are coded test variable values, air flow rate (litres / minute), packing density (grams / litre), particle size (mesh) and initial moisture content (percent) respectively. The significance of each coefficient was calculated by the t-test and p-values of the student and was reported in table 3. The greater is the magnitude of the t-value and the smaller is the p-value, the more the resulting coefficient was important. Values less than 0.05 for "Prob > F" suggest that the terms of the model were important.

In this case, important model terms for inulinase development were B, C, D, AB, AC, AD, BC A^2, B^2, C^2 and D^2. The terms of the model were not significant, showing values greater than 0.05. This implies that the square and linear effects of all the four parameters considered for the study were more important than the other effects. More significant also were the interactive effects of A–B and A–D.

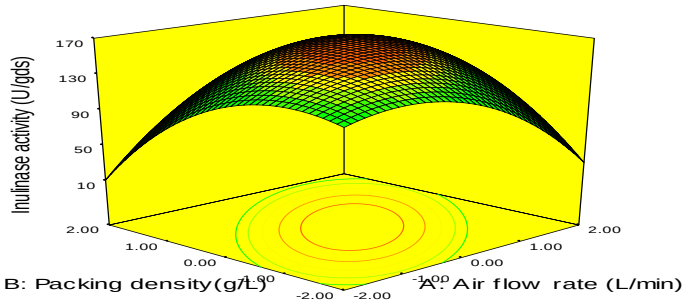

Fig. 1: 3D plot shows the effect of airflow rate and packing density on inulinase production.

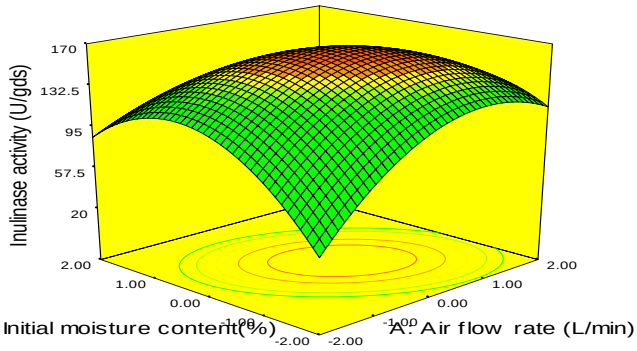

Fig. 2: 3D plot shows the effect of airflow rate and initial moisture content on inulinase production.

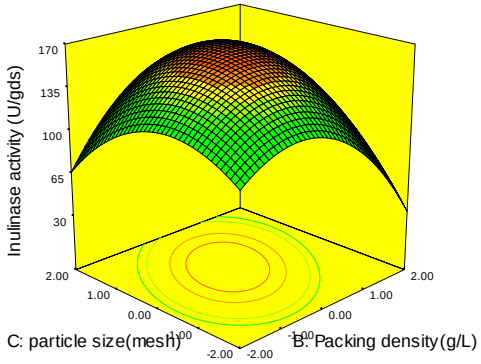

Fig. 3: 3D plot shows the effect of particle size and packing density on inulinase production.

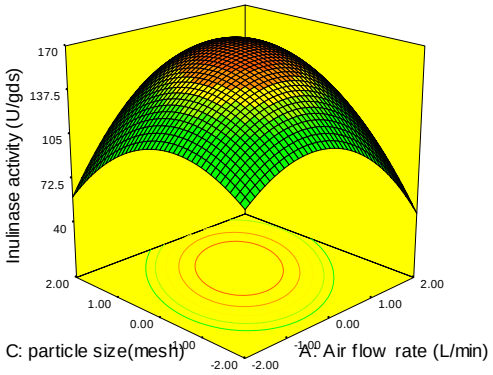

Fig. 4: 3D plot shows the effect of particle size and airflow rate on inulinase production.

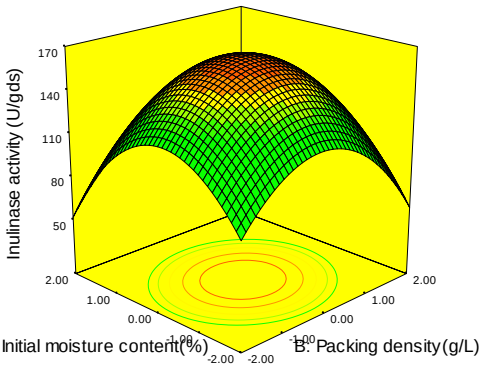

Fig. 5: 3D plot shows the effect of initial moisture content and packing density on inulinase production.

The contour and surface plot response were created for different interactions between any two independent variables, while keeping the value of the other variables as constant. These three-dimensional surfaces provide correct geometric representation and useful knowledge about the system's behavior within the experimental design. The response surface plots for inulinase synthesis by *Streptomyces sp.* using copra waste are seen in figs. 1–6.

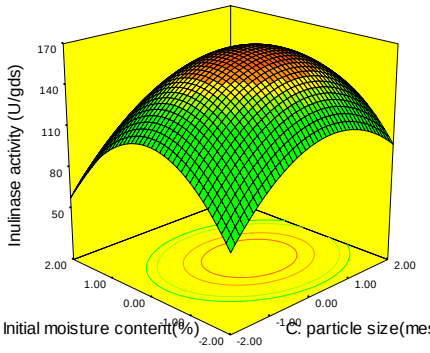

Fig. 6: 3D plot shows the effect of initial moisture content and particle size on inulinase production.

Fig. 1 shows the effects of air flow rate and packing density on inulinase synthesis. It was observed from the figure that the production of inulinase increases to 0.78 litres / minute and then decreases. It is because the aeration rate increases and allows the culture medium to decrease in temperature. Therefore, potential denaturalization of enzymes can be minimized. However, at higher airflow levels, the moisture content of the solid substrate was extremely reduced and thus the synthesis of enzymes was reduced. The optimum initial moisture content was 53 % clearly seen in fig 5. Similar findings were obtained[11]. They found impact of the airflow rate on the output of xylanase using a PBR. The highest enzyme activity was at intermediate flow rate but the activity decreased with higher aeration.

Likewise, high packing density limited the airflow and inhibited the growth of yeast. Minimum aeration was required so as not to affect the yeast's metabolic activity. The optimal density for the packaging was 45 grams / litre. Apple pomace in multilayer PBR obtained comparable results for the protein enrichment of lignocellulosic substrates and citric acid production[12,13]. The increase in particulate size decreases the output of inulinase. This can be explained by the fact that the substrate both serves as a source of nutrients and promotes microbial growth. Higher particles make transferring mass difficult due to reduced surface area and then nutrient availability[14]. This is clearly seen in figs. 3 and 4.

Optimum conditions were established for the highest inulinase production. In this study second order polynomial models obtained were used for each response in order to determine the specified optimum conditions. The quadratic sequential programming in MATLAB 7 was used to solve the equation for polynomial regression of second degree. The optimum values obtained by replacing the respective coded vector values are: air flow rate-0.78 litre / minute, initial moisture content-53%, packing density-45 grams / litre and particle size-8/10 mesh.

Experiments in the packed bed reactor were carried out under these controlled conditions. At optimized condition a maximum inulinase output of 176.4 U / gds was achieved. A

maximum inulinase activity of 165.4 U / gds was obtained in table 2, run no.6 and it was increased to 176.4 U / gds at optimized RSM level.

Conclusion

In this study, *Streptomyces sp*. was successfully employed by PBR for the production of inulinase. The considered four process parameters were optimized using RSM. The maximum inulinase activity in optimized condition was found to be 176.4 U / gds. This is greater than the un-optimized condition.

The results show near agreement between the experimental and predicted values obtained via RSM. Therefore, the RSM can be used effectively to increase inulinase production in PBR.

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