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Utilization of copra waste for the solid state fermentatative production of inulinase in batch and packed bed reactors

M. Dilipkumar[a,*], M. Rajasimman[a], N. Rajamohan[b]

[a] Department of Chemical Engineering, Annamalai University, Annamalainagar 608002, Tamilnadu, India
[b] Department of Chemical Engineering, Sohar University, Sohar, Oman

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ABSTRACT

In this study, screening and optimization of nutrients for inulinase production using copra waste has been studied. Plackett–Burman Design (PBD) was employed to screen the significant nutrients for inulinase production. Response surface methodology (RSM) was used to evaluate the effects of nutrient components in the medium. The second order regression equation provides the inulinase activity as the function of K_2HPO_4, $ZnSO_4{\cdot}7H_2O$ and soya bean cake. The optimum conditions are: K_2HPO_4 – 0.0047 g/gds, $ZnSO_4{\cdot}7H_2O$ – 0.02677 g/gds and soya bean cake – 0.06288 g/gds. At these optimized conditions, experiments were performed in packed bed bioreactor to optimize the process variables like air flow rate, packing density, particle size and moisture content. The optimum conditions were: air flow rate – 0.76 L/min, packing density – 38 g/L, particle size – 10/14 mesh and moisture content – 60%. At the optimized conditions, a maximum inulinase production of 239 U/gds was achieved.

1. Introduction

Industries like dairy, food and pharmaceuticals employ fats such as fructooligosaccharides and fructose as vital ingredients. Low calorific value and dietary fiber content make fructooligosaccharides to possess good nutritional properties. They have used as a replacement for sugars in many industries like confectionery and dairy. In addition to this, fructose is also a potential sweetening agent finding more utilization in the place of sucrose. This is attributed to the fact that sucrose is reported to cause atherosclerosis and cancer (Singh, Sooch, & Puri, 2007). Moreover, fructose is reported to enhance iron absorption in children and has a comparatively higher sweetening capacity. Interestingly, the production of both fructose and fructooligosaccharides are produced enzymatic hydrolysis (single step) of inulin using inulinase as the catalyst (Dilipkumar, Rajasimman, & Rajamohan, 2011a). Production of inulinase using different strains of microorganism like *Streptomyces* sp., *Kluyveromyces marxianus*, *Aspergillus niger*, *Bacillus smithi* have been reported (Laowklom, Chantanaphan, & Pinphanichakarn, 2012). Inulin is a polyfructan found in plant consisting of linear chains–β-(2,1)–linked fructose residues attached to a terminal sucrose molecule. Inulin is also found as a carbohydrate reserve in tuberous roots like chicory, dandelion and dahlia. It can be acid hydrolyzed at 80 °C and at low pH values leads to the destruction of fructose, a highly unfavored reaction. The complete hydrolysis of inulin by inulinase can result in 95% yield of pure fructose. With ultra-high fructose syrup productions strongly depending on the availability of inulinase, the requirement for high activity inulinase is very essential.

Solid-state fermentation (SSF) is generally defined as that in which microbial growth and product formation take place on a solid substrate in the absence of free water (Pandey, 2003). In the last two decades, SSF has received increasing interest partly because, as fermentation technology, it has lower energy requirements and produces less wastewater than submerged fermentation (Makino, Treichel, Mazutti, Maugeria, & Rodrigues, 2009). Utilization of cheaper, locally identified agro industrial residues is an attractive alternative for inulinase production, since the activity should be improved over, or at least remain the same as that obtained using a synthetic medium. The choice of the suitable microorganism will be more important for the successful production of this enzyme using agro waste as a substrate. In this research study, a cheap substrate, Copra waste, which is locally available, has been tried as a source for the production of inulinase using the Fungi, *Penicillium rugulosum.* Placket–Burman approach was implemented to screen the nutrients in the medium and a central composite design (CCD) was utilized for the optimization.

In solid state fermentation (SSF), packed bed reactors (PBR) are widely used for enzyme production (Cavalcanti, Gutarra, Freire, Castilho, & Sant'Anna Junior, 2005; Dilipkumar, Rajamohan, & Rajasimman, 2013; Mazutti et al., 2010a, 2010b, 2010c). The advantages of PBR are simplicity of construction and operation and the

* Corresponding author.
E-mail address: mdilip_kumar@yahoo.co.in (M. Dilipkumar).

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limitations are the difficulty in their control and optimization, owing to the limitations in mass and heat transfer. The objective of this work is to investigate the feasibility of producing inulinase in PBR. The performance of a PBR was investigated by manipulation variables such as air flow rate, packing density, particle size of the substrate and initial moisture content.

2. Materials and methods

2.1. Microorganism

Fungi, *P. rugulosum* (MTCC-3487) was purchased from Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. It was well preserved in the laboratory on solid medium at 5 °C. The medium composition was: Czapek concentrate 10.0 ml, K_2HPO_4 1.0 g, yeast extract 5.0 g, sucrose 30.0 g, and agar 15.0 g, distilled water 1.0 L. Composition of Czapek concentrate was: $NaNO_3$ – 30.0 g, KCl – 5.0 g, $MgSO_4{\cdot}7H_2O$ – 5.0 g, $FeSO_4{\cdot}7H_2O$ – 0.1 g and distilled water – 100.0 ml. Cells were harvested from slants and used to inoculate liquid medium.

2.2. Solid state fermentation

The substrate, Copra waste, was obtained from the Oil mill, Chidambaram, Tamilnadu, India. It was sundried for 48 h and then used as substrate. 10 g of substrate was taken in 250 ml Erlenmeyer flasks and the flask was supplemented with nutrients based on the experimental design. The moisture content of the media was adjusted to 60%. All the flasks were covered with hydrophobic cotton and autoclaved at 121 °C for 20 min. After cooling, each flask was inoculated with 2 ml of the suspension previously prepared and incubated for 120 h in a chamber with temperature and humidity control (Lark, India). In the preliminary screening process, the experiments were carried out for 5 days and it was found that at the 48th hour, the maximum production occurs. Hence experiments were carried out for 48 h.

2.3. Enzyme extraction procedure

After fermentation, distilled water was added (about 5 times) to the fermented matter and the contents were agitated for 30 min at 200 rpm on a rotary shaker (REMI, India) at 28 °C. Then the sample was centrifuged at 15 000 rpm for 20 min and the supernatant were analyzed by DNS method.

Table 1
Nutrient supplements for screening using Plackett–Burman design for inulinase production.

Variable		Levels, g/10 gds	
Nutrient code	Nutrient	Low (−1)	High (+1)
A	Yeast extract	0.01	0.05
B	Beef extract	0.05	0.15
C	$MnSO_4{\cdot}7H_2O$	0.10	0.50
D	K_2HPO_4	0.02	0.07
E	Soya bean cake	0.40	0.80
F	$MgSO_4{\cdot}7H_2O$	0.002	0.012
G	NH_4Cl	0.01	0.03
H	KCl	0.005	0.015
J	$(NH_4)_2HPO_4$	0.05	0.30
K	NH_4NO_3	0.05	0.10
L	$ZnSO_4{\cdot}7H_2O$	0.10	0.50
M	$(NH_4)_2SO_4$	0.06	0.10
N	Corn steep liquor	0.40	0.80
O	Peptone	0.05	0.15
P	Dextrose	0.10	0.30
Q	$FeSO_4{\cdot}7H_2O$	0.0005	0.002
R	KH_2PO_4	0.10	0.60
S	Urea	0.10	0.30

2.4. Experimental methods by response surface methodology (RSM)

RSM consist of a group of empirical techniques used for evaluation of relationship between cluster of controlled experimental variables and measured response. A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach. To determine which variables significantly affect inulinase production by *P. rugulosum*, Plackett–Burman design was used. Eighteen nutrients (Table 1) were screened in 20 experimental runs (Table 2) and insignificant ones were eliminated. The low level (−1) and high level (+1) of each factor are listed in Table 1. The statistical software package 'Design Expert 7.1.5' was used for analyzing the experimental data.

After the screening of nutrients, central composite design (CCD) was used to optimize the selected nutrients and to obtain a quadratic model. The selected nutrients were studied at five different levels (Table 3) and experiments were performed according to CCD shown in Table 3. The statistical software package 'Design Expert 7.1.5 was used to analyze the experimental data. Experiments were carried out in triplicate and the average inulinase activity was reported. A second order polynomial equation, which

Table 2
PBD for screening of important variables for inulinase production using *Penicillium rugulosum*.

Run no.	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	Inulinase, U/gds
1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	47.65
2	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	41.67
3	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	66.34
4	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	56.68
5	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	146.94
6	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	82.34
7	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	137.63
8	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	128.65
9	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	71.43
10	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	61.74
11	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	78.76
12	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	65.33
13	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	51.32
14	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	45.22
15	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	57.02
16	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	132.56
17	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	34.23
18	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	78.73
19	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	62.76
20	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	67.34

Table 3
CCD of factors in coded levels with enzyme activity as response.

Run no.	K_2HPO_4 (X_1)	$ZnSO_4{\cdot}7H_2O$ (X_2)	Soya bean cake (X_3)	Inulinase activity, U/gds	
				Experimental	Predicted
1	0.045	0.47	0.60	103.23	86.96
2	0.060	0.40	0.70	71.43	78.63
3	0.060	0.20	0.50	92.23	87.88
4	0.045	0.30	0.60	187.53	186.42
5	0.045	0.30	0.43	83.21	80.94
6	0.045	0.30	0.60	185.45	186.42
7	0.045	0.30	0.60	190.64	186.42
8	0.045	0.30	0.60	185.12	186.42
9	0.045	0.30	0.60	183.34	186.42
10	0.030	0.20	0.50	68.45	66.71
11	0.060	0.20	0.70	158.34	151.17
12	0.045	0.30	0.60	187.45	186.42
13	0.045	0.13	0.60	122.73	129.07
14	0.060	0.40	0.50	66.64	66.85
15	0.020	0.40	0.70	62.23	72.45
16	0.045	0.30	0.77	127.62	120.10
17	0.020	0.40	0.50	76.67	89.17
18	0.070	0.30	0.60	83.84	87.52
19	0.020	0.30	0.60	78.23	64.51
20	0.020	0.20	0.70	96.32	101.49

relates the response measured to the independent variables is used.

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

where Y is the inulinase activity, 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 statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

2.5. Enzyme assay

The concentration of reducing sugars released from inulin or sucrose was measured using the enzyme assays. The reaction mixture containing 1 ml of diluted crude enzyme and 4 ml of 2% inulin 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 (ELICO, India). A higher absorbance indicated a high level of reducing sugar produced and consequently, a high enzyme activity. One unit of inulinase activity (U) was defined as the amount of enzyme, which forms 1 μmol fructose per min. Results of the inulinase activity was represented in the units of activity/gram of dry substrate (U/gds.).

2.6. Packed bed reactor

A bench scale packed bed reactor made of Perspex, had a cylindrical shape of 4 cm diameter and 60 cm height was used. Sterile and moist air at the operating temperature enters at the bottom of the PBR and passes through the bed, exiting by the vessel top outlet. Air flow rate was measured with a rotameter (Placka, India). The substrate and the reactor were separately sterilized in autoclave (SELACO, India). Before packing the solid medium in the sterile reactor, it was inoculated with the microbes and its moisture content was adjusted based on the Box–Behnken design (BBD). The reactor was then filled with dry substrates, supplemented with the selected nutrients at their optimum conditions. The range and levels of process variables were given in Table 4. Optimization of the inlet air flow rate, packing density, particle size and initial moisture content was carried out by Box–Behnken design (Table 4). The experimental results were analyzed using the software Design Expert (7.1.5). After fermentation, the fermented solids are removed from the reactor and transferred to a glass flask and the inulinase was extracted.

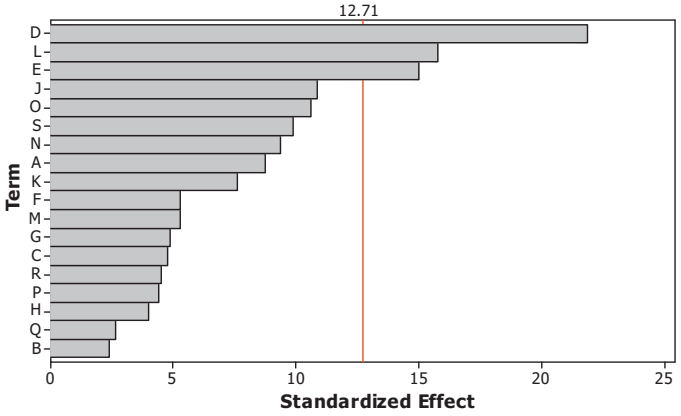

Fig. 1. Pareto chart showing the effect of media components on enzyme activity.

3. Results and discussion

3.1. Plackett–Burman design based screening of parameters

Plackett–Burman design provides a fast and an effective way to screen the important factors among several variables, thereby, saving time and not only maintaining but also convincing information from each parameter (Zhang et al., 2012). Experiments were carried out based on PBD and the results obtained were given in Table 2. From the table, it was observed that there is a wide variation in inulinase activity, which reflects the importance of optimization for the enhanced production of inulinase. From the Pareto chart (Fig. 1) the nutrients, K_2HPO_4, $ZnSO_4{\cdot}7H_2O$ and soya bean cake were found to be significant for the production of inulinase by *P. rugulosum* using copra waste. Hence these nutrients were selected for further optimization using CCD to maximize the production of inulinase.

Table 4
BBD based results for inulinase production in PBR.

Run no.	Air flow rate, L/min (A)	Packing density, g/L (B)	Particle size, mesh (C)	Moisture content, % (D)	Inulinase activity, U/gds	
					Experimental	Predicted
1	0.8	40	14/20	70	190.3	193.44
2	0.8	40	8/10	50	197.6	197.68
3	1.2	40	14/20	60	208.1	207.56
4	1.2	40	10/14	50	189.5	186.65
5	0.8	50	8/10	60	211.9	212.10
6	0.8	40	10/14	60	232.2	232.26
7	0.8	30	10/14	50	183.9	186.36
8	0.8	40	8/10	70	188.1	188.21
9	0.8	30	10/14	70	193.8	194.34
10	0.8	50	10/14	50	192.3	190.78
11	0.4	50	10/14	60	192.7	195.66
12	0.8	40	10/14	60	232.4	232.26
13	0.4	40	10/14	50	185.9	184.63
14	0.8	40	10/14	60	232.1	232.26
15	0.8	30	8/10	60	213.4	213.78
16	0.8	50	10/14	70	176.8	173.36
17	1.2	30	10/14	60	200.1	200.36
18	1.2	50	10/14	60	198.2	202.63
19	0.4	40	14/20	60	214.1	213.44
20	0.8	40	10/14	60	232.0	232.26
21	0.8	40	10/14	60	232.6	232.26
22	0.8	30	14/20	60	223.3	220.87
23	0.4	30	10/14	60	215.7	214.49
24	1.2	40	8/10	60	209.7	209.38
25	1.2	40	10/14	70	177.3	176.33
26	0.8	40	14/20	50	190.3	193.41
27	0.8	50	14/20	60	208.6	205.98
28	0.4	40	8/10	60	211.1	210.66
29	0.4	40	10/14	70	184.9	185.52

3.2. Optimization of nutrients for the inulinase production in batch reactor

RSM is a collection of mathematical and statistical techniques that are useful for modeling and analyzing problems which involved a response of interest influenced by several variables and the objective is to optimize this response (Montgomery, 2008; Soni, Singh, Kamble, & Banerjee, 2007). PBD and RSM have been successfully used to optimize some bioprocesses using the software of design expert (Ferreira, Duarte, Ribeiro, Queiroz, & Domingues, 2009; Mohana, Shrivastava, Divecha, & Madamwar, 2008). CCD was used to determine the optimum conditions for the inulinase production using *P. rugulosum*. Twenty experiments were performed at different combinations. The experimental and predicted values of inulinase activity were given in Table 3. The results were analyzed by analysis of variance (ANOVA). The second order regression equation gives the inulinase activity as the function of K_2HPO_4, $ZnSO_4{\cdot}7H_2O$ and soya bean cake. It was given as:

$$Y = 186.423 + 6.8397X_1 - 12.5178X_2 + 11.6424X_3 - 39.0346X_1^2 - 27.7208X_2^2 - 30.3725X_3^2 - 10.875X_1X_2 + 7.125X_1X_3 - 12.875X_2X_3 \quad (2)$$

where Y is the inulinase activity (U/gds), X_1, X_2 and X_3 are K_2HPO_4, $ZnSO_4{\cdot}7H_2O$ and soya bean cake, respectively.

Table 5 presents the ANOVA data for inulinase enzyme production. The Model F-value of 49.99 for inulinase production shows the significance of the model. Values of P less than 0.05 indicate that the model terms were significant and greater than 0.1 indicates that the model terms were not significant. In the present work, all the linear, interaction effects of X_1X_2, X_2X_3 and square effects of X_1, X_2 and X_3 were significant for inulinase production. The coefficient of determination (R^2) for inulinase activity was found to be 0.9783. The predicted R^2 value of 0.8373 was in reasonable agreement with the adjusted R^2 value of 0.9587. An adequate precision value greater than 4 is desirable. The adequate precision value of 17.007 indicates an adequate signal and suggests that the model can be used to navigate the design space.

The interactive effects of nutrients on inulinase production were studied by plotting 3D surface curves against any two independent variables, while keeping the other variables at its central (0) level. The 3D curves for the inulinase production were shown in Fig. 2(a) and (b). Fig. 2(a) shows the interactive effect of K_2HPO_4 and $ZnSO_4{\cdot}7H_2O$ on inulinase production. From figure it was inferred that the inulinase activity increases with increase in K_2HPO_4 concentration up to 0.0047 g/gds and thereafter inulinase activity decreases with further increase in K_2HPO_4. From the figure it was found that, increase in $ZnSO_4{\cdot}7H_2O$ resulted increase in inulinase activity upto 0.02677 g/gds, beyond this there was a decrease in inulinase activity. Fig. 2(b) shows that as soya bean cake concentration increases, the inulinase activity increases up to a value of 0.06288 g/gds after that the activity decreases. The optimum conditions for the maximum production of inulinase were determined by response surface analysis and also estimated by regression equation. The optimum conditions were: K_2HPO_4 – 0.0047 g/gds, $ZnSO_4{\cdot}7H_2O$ – 0.02677 g/gds and soya bean cake – 0.06288 g/gds. Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions. Three repeated experiments were performed and the results were compared. The inulinase activity obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model. At these optimized conditions, a maximum inulinase activity of 203 U/gds was observed.

3.3. Optimization of process variables in PBR for inulinase production

BBD was employed to optimize the process variables like air flow rate, packing density, particle size and initial moisture content for inulinase production in a PBR. Experiments were carried out based

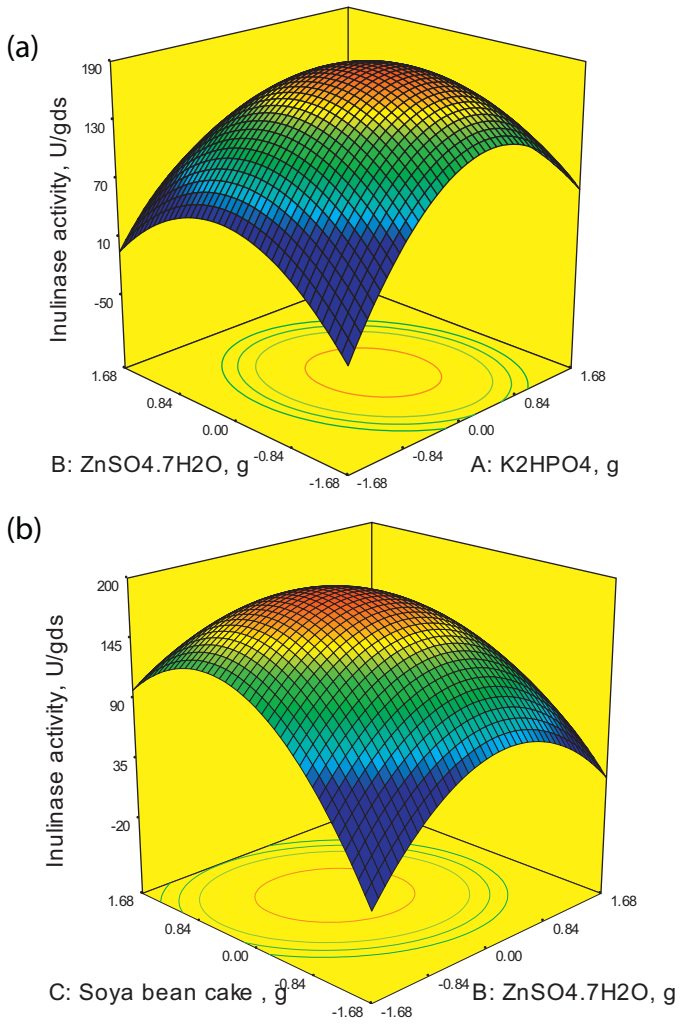

Fig. 2. (a) 3D plot showing the effect of K_2HPO_4 and $ZnSO_4{\cdot}7H_2O$ on inulinase activity. (b) 3D plot showing the effect of $ZnSO_4{\cdot}7H_2O$ and soya bean cake on inulinase activity.

on the BBD and the results obtained were given in Table 4. The second order polynomial equation for inulinase production obtained from RSM was

$$\text{Inulinase activity, U/gds} = 232.26 - 1.79\,A - 4.14\,B - 0.24\,C - 2.36\,D + 5.27\,AB + 1.15\,AC - 2.80\,AD + 3.30\,BC - 6.35\,BD - 2.38\,CD - 15.95\,A^2 - 13.03\,B^2 - 6.05\,C^2 - 33.03\,D^2 \quad (3)$$

The results were analyzed by analysis of variance (ANOVA) and were given in Table 5. The *F*-value (49.99) for model shows the significance of the model for inulinase production. From the *P* value it was inferred that the linear effects of air flow rate, packing density, moisture content, interactive effects of A–B, A–D B–C, B–D and square effects of air flow rate, packing density, particle size, moisture content, were significant for inulinase production. The coefficient of determination (R^2) for inulinase activity was found to be 0.9891. The predicted R^2 value of 0.9373 was in reasonable agreement with the adjusted R^2 value of 0.9782. The adequate precision value of 31.42 indicates an adequate signal and suggests that the model can be used to navigate the design space.

3.4. Effect of process variables in PBR for inulinase production

The interactive effects of process variables on inulinase production in PBR were studied by plotting 3D surface curves. Three dimensional surface plots give accurate geometrical representation and provide useful information about the behavior of the process within the experimental design. The 3D curves for the inulinase production in PBR were shown in Fig. 3(a and b). From Fig. 3(a), it was observed that increase in flow rate upto 0.76 L/min increases inulinase production and after that inulinase production decreases. The deleterious influence of the high aeration rates was possibly due to damaging effect of shear stress on microorganism and the reduction of moisture content of substrate which in turn could prevent microbial growth (Lonsane, Ghildyal, Budiatman, & Ramakrishna, 1985; Lu, Brooks, & Maddox, 1997; Mitchell, Pandey, Sangsurasak, & Krieger, 1999). Similar results were reported by Dilipkumar et al. (2013) for inulinase production and Milagres, Santos, Piovan, and Roberto (2004) for xylanase production using PBRs.

Table 5
ANOVA for the production of inulinase in batch reactor and PBR.

Bach reactor			Packed bed reactor		
Source	*F*	*P* > *F*	Source	*F*	*P* > *F*
Model	49.99	<0.0001	Model	90.65	<0.0001
X_1	6.22	0.0318	A	5.67	0.0320
X_2	20.82 18.01	0.0010	B	30.28	<0.0001
X_3	9.21	0.0017	C	0.10	0.7529
$X_1{*}X_2$	3.95	0.0126	D	9.82	0.0073
$X_1{*}X_3$	12.90	0.0749	AB	16.37	0.0012
$X_2{*}X_3$	213.66	0.0049	AC	0.78	0.3926
$X_1{*}X_1$	107.76	<0.0001	AD	4.61	0.0497
$X_2{*}X_2$	129.36	<0.0001	BC	6.41	0.0240
$X_3{*}X_3$		<0.0001	BD	23.73	0.0002
			CD	3.32	0.0899
			A^2	242.77	<0.0001
			B^2	161.90	<0.0001
			C^2	34.93	<0.0001
			D^2	1040.72	0.0007
Std. dev		10.14		2.61	
R^2		0.9783		0.9891	
Adj. R^2		0.9587		0.9782	
Pred. R^2		0.8373		0.9373	
C.V. %		8.44		1.28	
Adeq. precision		17.007		31.412	

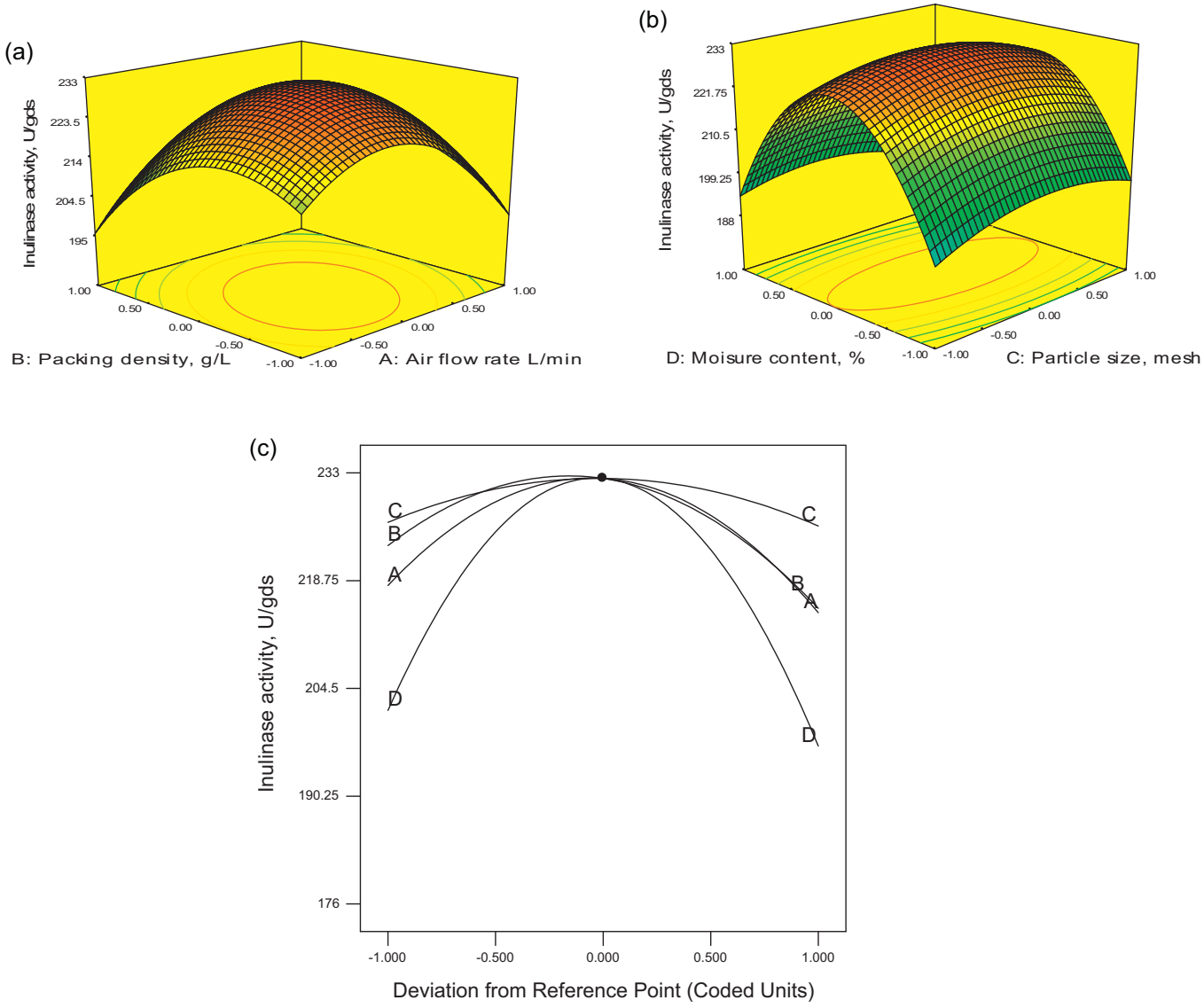

Fig. 3. (a) Interactive effect of air flow rate and packing density on inulinase production by *Penicillium rugulosum* using copra waste. (b) Interactive effect of particle size and moisture content on inulinase production by *Penicillium rugulosum* using copra waste. (c) Perturbation plot showing the effect of variables on inulinase production by *Penicillium rugulosum* using copra waste.

From Fig. 3(a), it was evident that packing density influenced the inulinase production. Although, aeration with low bed density enhanced the inulinase activity but higher aeration rate with excessive voidages inhibited its production due to shear stress in the packed bed. High packing density restricts the air flow and inhibited the microbial growth. Therefore, an optimum packing density was required to maintain the metabolic activity of the microorganism. From the plot it was found that a packing density of 38 g/L was optimum for maximum inulinase production. The results obtained in this study were comparable with the others reported for protein enrichment of lignocellulosic substrate and citric acid production from apple pomace in multi layer packed bed bioreactor (Shojaosadati & Babaeipour, 2002; Shojaosadati, Faraidouni, Madadi, & Mohamadpour, 1999).

Optimal particle size of substrate may be determined to obtain high yield of inulinase (Barrios-Gonzalez, Gonzalez, & Mejia, 1993; Pandey, 2003). The effect of particle size on inulinase production was shown in Fig. 3(b). From the figures it was found that the effect of particle size (considered in this study) on inulinase does not have significant variation. However at very small and large particle size, the production was found to be low. 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, Luccio, & Treichel, 2007). Hence the optimum particle size was found to be 10/14 mesh.

In SSF, initial moisture content of the substrate is a vital factor, because it influences microbial growth and enzyme production. Water present in the substrate makes the nutrients more easily accessible for cell growth and it has an impact on physico-chemical properties of the substrate. From the Fig. 3(b), it was inferred that the optimum initial moisture level was about 60% for copra waste. Substrate moistened at this level yielded a high inulinase activity. Lower or higher than 60% both decreases the inulinase production. High moisture levels reduced enzyme production as the substrate became waterlogged. Also high moisture content was known to reduce voidage or porosity of substrate, causes particles to stick together and adversely impacts oxygen transfer to the microorganism. In contrast, a low moisture level reduces water activity

to levels that are not conducive to supporting good microbial growth.

Fig. 3(c) shows the perturbation plot, which gives the comparative effects of the variables on inulinase production. A steep curvature in moisture content, D curve shows that the inulinase production was very sensitive to this variable. The comparatively semi-flat 'A' curve show less sensitivity of the inulinase production to alter with respect to a change in moisture content. The curve B was less sensitive when compared to the curves D and A. The particle size has no major impact in inulinase production when compared to other variables. It was clear from the perturbation plot that the most significant factor on the response was moisture content followed by air flow rate and packing density.

From the second order polynomial equation, the optimum conditions for the maximum production of inulinase were: air flow rate – 0.76 L/min, packing density – 38 g/L, particle size – 10/14 mesh and moisture content – 60%. The model was validated by carrying out experiments at these conditions. The inulinase activity obtained from experiments (239 U/gds) was very close to the actual response predicted by the regression model, which proved the validity of the model.

3.5. Comparison of inulinase production in batch and PBR

In batch reactor, only few works have been carried out for inulinase production using *P. rugulosum* and copra waste. A maximum inulinase activity of 268 U/gds was obtained by *P. rugulosum* using garlic as substrate (Dilipkumar, Rajasimman, & Rajamohan, 2011b). Copra waste has been used as a substrate for inulinase production. A maximum inulinase activity of 83.12 U/gds and 67.16 U/gds was obtained using *K. marxianus* and *Staphylococcus* sp., respectively (Selvakumar & Pandey, 1999).

In PBR, Mazutti et al. (2010a, 2010b, 2010c) employed *K. marxianus* for inulinase production under SSF. The substrate was a compost of sugarcane bagasse, molasses, corn steep liquor and soybean meal. They obtained a maximum yield of 463 and 436.7 U/gds in different operating conditions. Dilipkumar et al. (2013) used PBR for inulinase production by *K. marxianus* var. *marxianus* using pressmud as substrate. They achieved 300.5 U/gds inulinase activity. The inulinase activity obtained (239 U/gds) in this study are comparable with the others result.

4. Conclusions

The production of an enzyme by a bioprocess using an agro waste is a value addition to the agricultural residues. In this work, optimization of inulinase production by *P. rugulosum* using the cheap substrate copra waste was tried. The nutrients were screened using PBD and the selected nutrients were optimized using CCD to enhance the inulinase production. The best conditions for enzyme production are identified as K_2HPO_4 – 0.0047 g/gds, $ZnSO_4{\cdot}7H_2O$ – 0.02677 g/gds and soya bean cake – 0.06288 g/gds with respect to media components. In PBR, the effects of air flow rate, packing density, particle size and moisture content were studied using BBD. From the results it can be seen that, in packed bed reactor, inulinase production was increased by 18%. Maximum productivity obtained in this study was 239 U/gds, which was competitive to the reported literature to date.

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References

Barrios-Gonzalez, J., Gonzalez, H., & Mejia, A. (1993). Effect of size particle, packing density and agitation on penicillin production in solid state fermentation. *Biotechnology Advances*, *11*, 539–547.

Cavalcanti, E. A. C., Gutarra, M. L. E., Freire, D. M. G., Castilho, L. R., & Sant'Anna Junior, G. L. (2005). Lipase production by solid state fermentation in fixed bed bioreactors. *Brazilian Archives of Biology and Technology*, *48*, 79–84.

Dilipkumar, M., Rajasimman, M., & Rajamohan, N. (2011a). Application of statistical design for the production of inulinase by *Streptomyces* sp. using pressmud. *Frontiers of Chemical Science and Engineering*, *5*, 463–470.

Dilipkumar, M., Rajasimman, M., & Rajamohan, N. (2011b). Optimization of media component in inulinase production using garlic by *P. rugulosum*. *Journal of Advanced Scientific Research*, *2*, 24–33.

Dilipkumar, M., Rajamohan, N., & Rajasimman, M. (2013). Inulinase production in a packed bed reactor by solid state fermentation. *Carbohydrate Polymers*, *96*, 196–199.

Ferreira, S., Duarte, A. P., Ribeiro, M. H. L., Queiroz, J. A., & Domingues, F. C. (2009). Response surface optimization of enzymatic hydrolysis of *Cistusladanifer and Cytisusstriatus* for bioethanol production. *Biochemical Engineering Journal*, *45*, 192–200.

Laowklom, N., Chantanaphan, R., & Pinphanichakarn, P. (2012). Production, purification and characterization of inulinase from a newly isolated *Streptomyces* sp. CP01. *Natural Resources*, *3*, 137–144.

Lonsane, B. K., Ghildyal, N. P., Budiatman, S., & Ramakrishna, S. V. (1985). Engineering aspects of solid state fermentation. *Enzyme and Microbial Technology*, *7*, 258–265.

Lu, M., Brooks, J. D., & Maddox, I. S. (1997). Citric acid production by solid state fermentation in a packed-bed reactor using *Aspergillus niger*. *Enzyme and Microbial Technology*, *21*, 392–397.

Makino, Y., Treichel, H., Mazutti, M. A., Maugeria, F., & Rodrigues, M. I. (2009). Inulinase bio-production using agroindustrial residues: Screening of microorganisms and process parameters optimization. *Journal of Chemical Technology and Biotechnology*, *84*, 1056–1062.

Mazutti, M., Ceni, G., Luccio, M. D., & Treichel, H. (2007). Production of inulinase by solid state fermentation: Effect of process parameters on production and preliminary characterization of enzyme preparations. *Bioprocess and Biosystems Engineering*, *30*, 297–304.

Mazutti, M. A., Zabot, G., Boni, G., Skovronski, A., de Oliveira, D., Di Luccio, M., et al. (2010b). Kinetics of inulinase production by solid state fermentation in a packed bed bioreactor. *Food Chemistry*, *120*, 163–173.

Mazutti, M. A., Zabot, G., Boni, G., Skovronski, A., de Oliveira, D., Di Luccio, M., et al. (2010c). Optimization of inulinase production by solid state fermentation in a packed-bed bioreactor. *Journal of Chemical Technology and Biotechnology*, *85*, 109–114.

Mazutti, M. A., Zabot, G., Boni, G., Skovronski, A., Oliveira, D., di Luccio, M., et al. (2010). Mathematical modeling of *Kluyveromyces marxianus* growth in solid state fermentation using a packed-bed bioreactor. *Journal of Industrial Microbiology and Biotechnology*, *37*, 391–400.

Milagres, A., Santos, E., Piovan, T., & Roberto, I. (2004). Production of xylanase by *Thermoascus aurantiacus* from sugar cane bagasse in an aerated growth fermentor. *Process Biochemistry*, *39*, 1387–1391.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.

Mitchell, D. A., Pandey, A., Sangsurasak, P., & Krieger, N. (1999). Scaleup strategies for packed-bed bioreactors for solid state fermentation. *Process Biochemistry*, *35*, 167–178.

Mohana, S., Shrivastava, S., Divecha, J., & Madamwar, D. (2008). Response surface methodology for optimization of medium for decolorization of textile dye Direct Black 22 by a novel bacterial consortium. *Bioresource Technology*, *99*, 562–569.

Montgomery, D. C. (2008). *Design and analysis of experiments* (7th ed.). New York, NY, USA: John Wiley & Sons.

Pandey, A. (2003). Solid state fermentation. *Biochemical Engineering Journal*, *13*, 81–84.

Selvakumar, P., & Pandey, A. (1999). Solid state fermentation for the synthesis of inulinase from *Staphylococcus* sp. and *Kluyveromyces marxianus*. *Process Biochemistry*, *34*, 851–855.

Shojaosadati, S. A., & Babaeipour, V. (2002). Citric acid production from apple pomace in multi-layer packed bed solid-state bioreactor. *Process Biochemistry*, *37*, 909–914.

Shojaosadati, S. A., Faraidouni, R., Madadi, N. A., & Mohamadpour, I. (1999). Protein enrichment of lignocellulosic substrates by solid state fermentation using *Neurospora sitophila*. *Resources, Conservation and Recycling*, *27*, 73–87.

Singh, S. R., Sooch, B. S., & Puri, M. (2007). Optimization of medium and process parameters for the production of inulinase from a newly isolated *Kluyveromyces marxianus* YS-1. *Bioresource Technology*, *98*, 2518–2525.

Soni, P., Singh, M., Kamble, A. L., & Banerjee, U. C. (2007). Response surface optimization of the critical medium components for carbonyl reductase production by *Candida viswanathii* MTCC 5158. *Bioresource Technology*, *98*, 829–833.

Uzunova, K., Vassileva, A., Ivanova, V., Spasova, D., & Tonkova, A. (2002). Thermostable exo-inulinase production by semicontinuous cultivation of membrane-immobilized *Bacillus sp.*11 cells. *Process Biochemistry*, *37*, 863–868.

Zhang, Y., Wang, Y., Wang, Z. G., Wang, X., Guo, H. S., Meng, D. F., et al. (2012). Optimization of fermentation medium for the production of atrazine degrading strain *Acinetobacter* sp. DNS3 2 by statistical analysis system. *Journal of Biomedicine and Biotechnology*, 7 pp (Article ID 623062).