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ORIGINAL ARTICLE

Response surface optimization of solid state fermentation for inulinase production from *Penicillium oxalicum* using corn bran

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Abstract Response surface methodology has been implemented for the utilization of corn bran for inulinase production by *Penicillium oxalicum*. CCRD of RSM with 15 runs was practiced to optimize three independent variables: moisture (70–90%), incubation time (4–8 days) and pH (5–8). However, other media constituents viz. inulin (1%), $NaNO_3$ (0.2%), $NH_4H_2PO_4$ (0.2%), KH_2PO_4 (0.2%), $MgSO_4{\cdot}7H_2O$ (0.05%) and $FeSO_4{\cdot}7H_2O$ (0.001%) were kept constant during solid state fermentations. Solid state fermentations were carried out at 30 °C at flask-level. A substantial inulinase production (77.95 IU/gds) was obtained under the optimized conditions i.e., moisture (80%), incubation time (6.0 days) and pH (6.5). Multiple correlation coefficient 'R^2' for inulinase production was 1.00, which justifies good agreement between experimental and predicted values. Besides, 'R^2' value close to one, also authenticates the validity of the model. The experimentation carried out at laboratory scale shown corn bran a good substrate for inulinase production by *P. oxalicum*.

Keywords *Penicillium oxalicum* · Inulinase · Corn bran · Solid state fermentation

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Introduction

Out of innumerable industrial enzymes, inulinases are important and valuable enzymes used for high fructose syrup (Singh and Chauhan 2016; Singh et al. 2018) and fructooligosaccharides production (Singh and Singh 2010; Singh et al. 2016). Inulinases are inducible enzymes which require an inducer for their synthesis. Inulin is considered as the most effective substrate as well as an inducer for their production. However, inulin is an expensive substrate. Owing to the cost of pure inulin, various inulin-rich raw materials are becoming an alternative choice as substrate for inulinase production. Various low-cost agro-industrial residues such as artichoke leaves, press mud (a sugar industry waste), sugarcane bagasse, soybean bran, wheat bran, etc. have also been reported as potent substrates for inulinase production (Singh and Chauhan 2016). Inulinases can be produced by either submerged fermentation (SmF) or solid state fermentation (SSF). In recent years, inulinase production by SSF has gained enormous attention over SmF, due to its some advantageous features like utilization of unexploited biotic resources, natural growth conditions to the growing cells, low production cost, reduced energy consumption, use of simple technique, less waste water generation, good product recovery, etc. Moreover, it also gives higher metabolite productivity by reducing catabolite repression (Pandey 2003; Bhargav et al. 2008).

The assessment of fermentation conditions and type of microbial source used for the production of a particular metabolite are of immense importance, since they significantly affect the product yield. Different strains of *Aspergillus* sp., *Penicillium* sp., *Kluyveromyces* sp., *Bacillus* sp., etc. have been reported as efficient inulinase producers (Singh et al. 2013; Singh and Chauhan 2016). However for SSF, filamentous fungi are the perpetual choice owing to

their physiological, biochemical and enzymological properties. Moreover, their ability to tolerate low water activity (A_w) and to easily thrive in high osmotic conditions, makes filamentous fungi a good candidate for the bioconversion of substrate in SSF (Mienda et al. 2011). Corn bran is an agro-industrial residue rich in carbohydrates (78%), proteins (3.5%), iron (16%), fats (1%) etc. (Honig and Rackis 1979). After dry-grinding of corn, its bran is generally regarded as a waste or used as a livestock feed. Considering the requirement for the development of a cost-effective substrate for inulinase production, corn bran can be combined with a minute quantity of inulin (as an inducer) for inulinase production in SSF. Therefore, the present study was carried out to optimise solid state fermentation of corn bran for inulinase production by *Penicillium oxalicum* BGPUP-4 using response surface methodology (RSM). Literature survey reveals, this is the first report on inulinase production from corn bran by *Penicillium oxalicum* BGPUP-4, under SSF.

Materials and methods

Fungal isolate

Penicillium oxalicum BGPUP-4, an isolate of our laboratory was used in the present investigation. The culture was maintained on potato dextrose agar (PDA) slopes as described earlier (Singh and Chauhan 2017) and culture slopes were stored at 4 °C, until further use.

Preparation of inoculum

Inoculum was prepared on PDA plates. A loopful of fungal stock culture was inoculated in PDA plates and incubated at 30 °C for 5 days for fungal growth (Singh and Chauhan 2017). Thereafter, fungal spores were harvested by flooding the PDA plate containing fungal culture with sterile saline containing 0.01% (v/v) Tween 80 (Trivedi et al. 2012). Then, spores were displaced from the hyphae using a sterile glass spreader. Afterwards, remained hyphal fragments were removed by filtering spore suspension through sterilized absorbent cotton wool plugs. The number of spores were counted using a haemocytometer (Erma, Japan) and microscope (Olympus, India), and the spore count was adjusted to 1×10^4 spores/mL, under aseptic conditions.

Solid state fermentation (SSF)

Corn bran procured from a flour mill, Patiala, India was grounded in a grinder. Its uniform particle size was maintained by passing it through a 150 µm sieve. Fermentations were carried out in Erlenmeyer flasks (250 mL) containing 10 g corn bran supplemented with media ingredients containing (%, w/v): inulin 1 (as an inducer), $NH_4H_2PO_4$ 0.2, $NaNO_3$ 0.2, KH_2PO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.05 and $FeSO_4{\cdot}7H_2O$ 0.001. Literature survey on solid state fermentation for the production of industrial enzymes revealed that moisture, pH and fermentation time have strong influence during SSF (Pandey et al. 1999; Krishna 2005). Therefore, three independent variables viz., A: moisture (70–90%), B: incubation time (4.0–6.0 days) and C: pH (5.0–8.0), were selected for optimization using RSM, whereas aforementioned medium components were kept constant during the fermentations. The Erlenmeyer flasks plugged with hydrophobic cotton were sterilized at 121 °C for 30 min. Preliminary studies showed no change in moisture content of the substrate after sterilization. After sterilization and cooling of the medium, 2 mL (1×10^4 spores/mL) of spore suspension was inoculated and mixed intermittently using a sterile glass rod. Subsequently, each flask was incubated at 30 °C, under static conditions.

Experimental design for the optimization of inulinase production from *P. oxalicum* BGPUP-4 using corn bran as solid substrate

In the present study, experiments for the optimization of three independent variables (moisture, incubation time and pH) were conducted using CCRD of RSM. Each independent variable was studied at five coded levels: − 1.414, − 1, 0, 1 and 1.414 (Table 1). A total 15 runs with different combinations was resulted, out of which four were factorial, six axial points and five replicates at the centre point. The experimental runs 1, 7, 9, 12 and 14 at the centre point were used to determine the duplicity of the model. All the experiments were accomplished in triplicates. Furthermore, validation experiments were also performed to verify the model's verity by comparing predicted values with experimental data.

Design expert version 7.0.0 software package (State-Ease Inc., Minniapolis, MN, USA) was used for statistical analysis. A 2^3 factorial design was used for the optimization of three independent variables. Using least square

Table 1 Values of coded levels used for the experimental design

Independent variables	Code	Actual levels of coded factors				
		− 1.414	− 1	0	1	1.414
Moisture content (%)	A	66.0	70	80	90	94.0
Incubation time (days)	B	4.0	4	6	8	9.0
pH	C	5.0	5	6.5	8	8.6

method, the following second-order polynomial equation was fitted for the analysis of experimental data of each independent variable:

$$Y = \beta_0 + \Sigma\beta iXi + \Sigma\beta_{ii}Xi^2 + \Sigma\beta_{ij}XiXj \quad (1)$$

where Y is measured response, β_0 is the intercept term, βi is linear coefficient, β_{ii} is quadratic coefficient, β_{ij} is an interaction coefficient and X_i and X_j are coded input variables. Student's t test and Fisher's F test were also utilized for the confirmation of statistical significance of regression coefficients and second-order model equation and model terms. Moreover, the statistical significance of the polynomial model was justified using analysis of variance (ANOVA). Additionally, quality of the fitted polynomial model was expressed using coefficient of determination R^2, which exhibits variability in the observed response and interactions between different variables. Furthermore, response surface 3D graphs were also evaluated to study interactions between the variables and their resultant effect on inulinase production.

Inulinase extraction

Inulinase extraction was carried out by adding 100 mL of sodium acetate buffer (0.1 M, pH 5.0) to the fermented substrate in each Erlenmeyer flask. Each flask was kept under agitation (150 rpm) on a rotary shaker (CIS-24 BL, REMI, Mumbai, India) for 2 h at 30 °C. Then, the extract was filtered using Whatman filter paper No. 1 followed by centrifugation at 5000 rpm for 20 min at 4 °C. Supernatant was separated from pellet and assayed for inulinase activity (Mazutti et al. 2006).

Inulinase assay

Inulinase assay was carried out by preparing a reaction mixture containing 0.1 mL of crude enzyme extract with 0.9 mL of inulin solution (2% prepared in sodium acetate buffer, 0.1 M, pH 5.0). Reaction mixture was incubated at 55 °C for 10 min. Thereafter, reaction was stopped by denaturing enzyme in reaction mixture in a boiling water bath for 10 min. Reducing sugars in hydrolysate were analysed by 3,5 dinitrosalicylic method (Miller 1959). One unit of inulinase is defined as the amount of enzyme that produces one micromole of fructose per minute, under standard assay conditions.

Results and discussion

Statistical analysis of the model using analysis of variance (ANOVA)

Moisture, incubation time and pH were selected as independent variables for the statistical optimization of inulinase production from corn bran by *P. oxalicum* BGPUP-4. CCRD was used to calculate the experimental design matrix. Different statistical models i.e., linear, 2F1, quadratic and cubic, were analysed to find the best fitted model for the generation of regression equation of the experimental data. Quadratic model (a suitable model), due to high F value was evaluated using sequential sum of squares and ANOVA. The model's higher F value (2.577E+005) for inulinase production justifies the significance of the model. Table 2 elaborates the experimental design and the results obtained for the response after statistical optimization. Second order polynomial equation was used to signify the fitness of the model. The values of regression coefficients were calculated and the following fitted second-order polynomial equation (in terms of coded values) for predicting inulinase production, regardless of coefficient's significance is as given below:

$$\begin{aligned}\text{Inulinase production} = {} & +77.88 + 2.68^{*}\text{A} - 7.65^{*}\text{B} - 0.75 \\ & {}^{*}\text{C} - 1.42^{*}\text{A}^{*}\text{B} - 4.42^{*}\text{A}^{*}\text{C} \\ & + 3.10^{*}\text{B}^{*}\text{C} - 28.02^{*}\text{A}^2 - 21.60^{*}\text{B}^2 \\ & - 15.92^{*}\text{C}^2\end{aligned} \quad (2)$$

where A, is moisture; B, incubation time and C, pH.

The statistical significance of Eq. 2 for selected quadratic model was affirmed using ANOVA. Fisher's F test and student's t test were also used to uphold the relevance of independent variables with 95% confidence level. Higher F value and smaller Prob > F value demonstrates significance of the corresponding coefficient term (Singh and Singh 2014). Besides, coefficient term with Prob > F value very small (less than 0.05 by default) also shows that the sources has been tested substantially and can be considered as significant model terms (Table 3). Model F value of 2.577E+005 infers that the model is significant. There was only 0.01% chance for the occurrence of this large value due to noise. In the present model, model terms: A, B, C, AB, AC, BC, A^2, B^2 and C^2 were determined as significant for inulinase production ($p > 0.0001$). Degree of freedom for pure error is used to express replicate or repeated runs. Each value supplements − 1 degree of freedom for pure error. Therefore, degree of freedom between 3 and 4 shows significance of the model. Furthermore, its value 4 for inulinase production for the

Table 2 Central composite rotatable design to study the effect of three independent variables on inulinase production from *P. oxalicum*

Run	A	B	C	Experimental inulinase production (IU/gds)	Predicted inulinase production (IU/gds)
1	80.0	6.0	6.5	77.85	77.89
2	70.0	4.0	5.0	15.36	15.33
3	94.0	6.0	6.5	25.61	25.64
4	90.0	4.0	8.0	15.86	15.83
5	70.0	8.0	8.0	10.25	10.22
6	80.0	6.0	5.0	47.08	47.11
7	80.0	6.0	6.5	77.95	77.89
8	90.0	8.0	5.0	8.05	8.02
9	80.0	6.0	6.5	77.82	77.89
10	80.0	9.0	6.5	23.83	23.86
11	80.0	6.0	8.6	44.96	44.99
12	80.0	6.0	6.5	77.94	77.89
13	80.0	4.0	6.5	45.46	45.49
14	80.0	6.0	6.5	77.92	77.89
15	66.0	6.0	6.5	18.03	18.06

Symbols A, B and C are same as designated in Table 1

IU/gds: International units/gram of dry substrate

Table 3 Analysis of variance (ANOVA) and regression analysis of the quadratic model for inulinase production

Source*	ANOVA			Regression analysis		
	Sum of squares	df	Probability > F	Coefficient estimate	Standard error	F value
Model/intercept	11203.13	9	< 0.0001	77.88	0.030	2.577E+005
A	28.73	1	< 0.0001	2.68	0.035	5947.87
B	233.93	1	< 0.0001	− 7.65	0.035	48432.39
C	2.25	1	< 0.0001	− 0.75	0.035	465.26
AB	4.06	1	< 0.0001	− 1.42	0.049	840.29
AC	39.03	1	< 0.0001	− 4.42	0.049	8079.95
BC	19.28	1	< 0.0001	3.10	0.049	3991.97
A^2	6054.84	1	< 0.0001	− 28.02	0.025	1.254E+006
B^2	3600.29	1	< 0.0001	− 21.60	0.025	7.454E+005
C^2	1954.32	1	< 0.0001	− 15.92	0.025	4.046E+005
Residual	0.024	5				
Lack of Fit	0.011	1	0.1457			
Pure error	0.013	4				
Cor total	11,203.15	14				

*Symbols A, B and C are same as designated in Table 1

present polynomial model authenticates the model's validity and significance. Replicate runs represent actual error in the model as only random variations cause differences in the observed responses. "Lack of fit" test is important to demonstrate functional relationship between experimental and response variables. Insignificant "Lack-of-fit" is virtuous. In the present model, "Lack of fit" was 3.25, which implies insignificant "Lack of fit" relative to the pure error, justifying the fitness of the model.

Multiple correlation coefficient 'R^2' was used to correlate variable's actual and predicted values and demonstrate the goodness of fit of the model (Table 4). A regression model with higher 'R^2' value reflects a very good correlation. Therefore, the 'R^2' value 1.00 for inulinase production indicates good fitness between experimental and

Table 4 Goodness of fit of the model

Source	Value	Source	Value
Std. deviation	0.059	R^2	1.00
Mean	28.71	Adjusted R^2	1.00
C.V.%	0.20	Predicted R^2	0.99
Press	0.092	Adeq. precision	1231.21

predicted values in the present model. Besides, it also shows that approximately 100% variability in the response could be explained by this model and the model is reliable for predicting inulinase production. Moreover, the values of adjusted R^2 (1.00) and predicted R^2 (0.99) are within the difference of 0.2 with each other, representing the reasonable agreement between the two values, error free data and model selection. A lower value of coefficient of variation (C.V.% = 0.20) specifies a better precision and reliability of the experiments. Adequate precision measures the signal to noise ratio, wherein a ratio greater than 4 is desirable and designate adequate model discrimination. Therefore the ratio 1231.21 for inulinase production defines an adequate signal for the present model and predicts that the model can be used to navigate the design space.

Interactions of solid state fermentation variables for inulinase production from corn bran

The effect of different combinations of three independent variables was interpreted using 3D response surface graphs (Fig. 1a–c). Three dimensional surface curves helped in accurate geometrical analysis, provided significant information regarding experimental design and signified the role played by each factor and the effect of their interactions on the response under SSF. Although submerged fermentation (SmF) is a preferred technology for the production of enzymes, a significant interest in using SSF for the production of wide range of industrial enzymes has arisen eventually (Pandey et al. 1999). SSF offers several potential advantages for the production and bioprocessing of innumerable value-added products. Amongst various advantages of SSF over SmF, higher enzyme titer and low catabolic repression are the most desirable properties. Besides, in SSF agro-industrial residues are generally used for enzyme production, consequently a cost-effective method is practised. Substrate, moisture level, fermentation time, aeration and ionic balance are important factors affecting SSF (Pandey et al. 1999). Moisture 80%, incubation time 6.0 days and pH 6.5 were found optimal for inulinase production (77.95 IU/gds) from *P. oxalicum* BGPUP-4 in SSF. Moisture content higher and lower than 80%, supported less inulinase production. Moisture complexed with in solid substrate or present as a thin layer either adsorbed on the surface of a substrate or bound within the capillary regions of the substrate has a significant effect on fungal conidial induction, metabolite production and growth kinetics under SSF (Pandey et al. 1999). However, moisture requirement varies from species to species. Generally, minimum 20% of moisture level is considered essential for facilitating nutrient absorption and fungal growth (Nuñez-Gaona et al. 2010). A raised moisture level can cause filling of the substrate pores, which diminishes oxygen mass transfer coefficient, consequently preventing hyphal growth and desired metabolite production (Pandey 2003). The maximal fungal growth and inulinase production at 80% moisture has also been reported from *Aspergillus ficuum* (Chen et al. 2011) and *A. tubingensis* (Trivedi et al. 2012), whereas maximum inulinase production from *Pichia guilliermondii* has been reported at 60.5% moisture level (Guo et al. 2009). This shows that moisture level varies for different species for metabolite production by SSF. In fungal solid state fermentations, the hyphal mode of growth of filamentous fungi aids them to penetrate easily into the solid substrate during SSF. The cell wall structure attached to the tip and mycelial branching ensures a firm structure. Unlike in SmF, hydrolytic enzymes excrete at the hyphal tip without getting diluted. Consequently, making hydrolytic action of enzyme more efficient and allowing penetration of hyphal tips in most of the solid substrates. Incubation period is also a very crucial factor for enzyme biosynthesis. Maximum inulinase production from *P. oxalicum* BGPUP-4 was observed after 6.0 days of fermentation. After that, low inulinase yield was observed. Reduction in inulinase production after 6.0 days of incubation can be accredited either to secretion of proteases (denature the enzyme by hydrolysing its peptide bonds) or diminution of nutrients in the medium. Our results corroborate the findings on inulinase production from *Bacillus safensis* (Singh et al. 2013), *P. expansum* (Fernandes et al. 2012) and *K. marxianus* (Singh et al. 2007). Medium pH is also significant in channelizing proper microbial growth and metabolite production. At pH 6.5 with moisture level 80% and incubation time 6.0 days, maximal inulinase production (77.95 IU/gds) was obtained. Thereafter, decline in inulinase production was observed. A very minute change in ionic

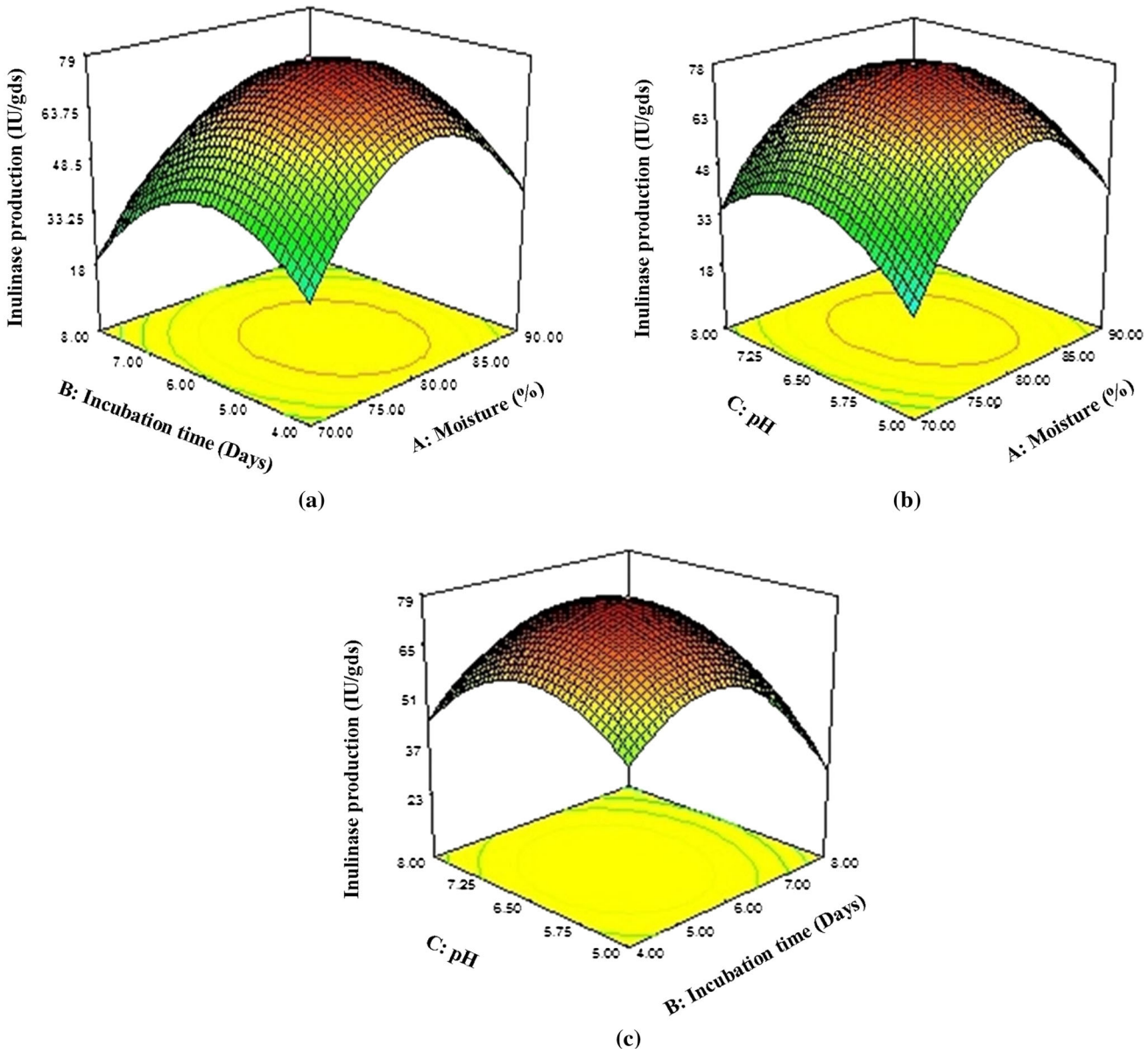

Fig. 1 Response surface 3-D graphs showing the effect of: **a** moisture and incubation time; **b** moisture and pH; and **c** incubation time and pH on inulinase production from corn bran by *P. oxalicum*

strength of the medium can affect surface charge distribution on enzyme, which subsequently effects its production. Most of the fungal species produce inulinase in slightly acidic environment (Singh and Chauhan 2016). Studies on inulinase production from *K. marxianus* (Makino et al. 2009), *P. guilliermondii* (Guo et al. 2009), *P. oxalicum* (Singh et al. 2017) and *A. niger* (Dinarvand et al. 2017) corroborates our findings on inulinase production from corn bran by *P. oxalicum* BGPUP-4. However, the measurement and control of this variable is very difficult in SSF. The agro-industrial substrates used in SSF itself have buffering effect due to their complex composition, but sometimes a mixture of nitrogen sources is used to maintain pH of the medium during SSF (Pérez-Guerra et al. 2003).

The interactive effect of three variables are depicted in Fig. 1a–c. Moisture content 80%, incubation time 6.0 days and pH 6.5 were found optimal for obtaining maximum inulinase production (77.95 IU/gds) from *P. oxalicum* BGPUP-4. An appropriate moisture level is the most important factor for fungal conidial induction and metabolite production. Raised moisture content diminishes oxygen mass transfer coefficient hindering biochemical events significant for metabolite production, whereas very low moisture content may not be sufficient to meet the lowest moisture requirement of the cells to thrive and

initiate hyphal progression (Pandey 2003). The three variables were observed interdependent on each other in contributing maximum inulinase yield. Moisture level at 80% and 6.0 days of incubation were most suitable for supporting fungal growth and inulinase production. After 6.0 days of fermentation, reduction in inulinase production was observed, which can be accredited to change in moisture level of the medium with time, secretion of proteases and diminution of nutrients. Moreover, pH 6.5 of the medium observed to show maximal inulinase production with moisture level 80% and incubation of 6.0 days. Fermentation time subtly effects pH of the medium. With the progress in fermentation, medium pH may be altered due to the accumulation of secondary metabolites, which can change the ionic environment of the medium. This may affect the cells growth and desired metabolite production. A decrease in inulinase production from 77.95 to 10.25 (IU/gds) was observed at very high pH (8.0), which suggests the interdependency of three variables effecting inulinase production.

Enzyme production is a fast growing field in biotechnology. Amongst important industrial enzymes, enzymes used in food fermentation technology are gaining continuous momentum and attention. Inulinases are also one of the important class of such enzymes which can be used for the production of high fructose syrup (HFS) and fructooligosaccharides (FOSs). HFS is an important sweetener and food ingredient, whereas FOSs have gut-stimulating properties. In the present study, a cost-effective substrate has been used for the production of inulinase. Corn bran can be a good candidate for the production of inulinase in scale-up studies. The produced enzyme can further be explored for the production of HFS and FOSs.

Validation of experimental model

To examine the validation of the quadratic model for predicting the inulinase production from *P. oxalicum* BGPUP-4, experimental trials were conducted in triplicates using optimized conditions (moisture 80%, incubation time 6.0 days and pH 6.5). The maximum inulinase production obtained from the performed experiments was 77.95 (IU/gds). On comparing the experimental data with predicted values of the regression model, validation of the present polynomial model for inulinase production from *P. oxalicum* BGPUP-4 under SSF can be easily assessed. Furthermore, R^2 value close to one justifies the good agreement between actual and predicted results, which also authenticates the validity of the present model.

Conclusion

The present model elaborated the pertinency of response surface methodology as an effective tool for carrying out simultaneous optimization of different variables for inulinase production from corn bran by *P. oxalicum* BGPUP-4 under SSF. After optimization, a significant 1.94-fold (data not shown) increase in inulinase production was observed. On the basis of ANOVA, the quadratic model was inferred substantial in displaying the effect of interaction among different input variables on inulinase production. The results obtained for inulinase production at flask-level solid state fermentations, encourages that corn bran can be explored as a substrate for the production of inulinase in scale-up studies.

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