Sequential statistical optimization of lactose-based medium and process variables for inulinase production from *Penicillium oxalicum* BGPUP-4

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Abstract

A statistical tool of response surface methodology was used sequentially to optimise lactose-based medium and process variables for inulinase production from *Penicillium oxalicum* BGPUP-4. Two-level CCRD with four variables, for each design, was used for the optimization study. The independent variables: lactose 1–3%, $\text{NH}_4\text{H}_2\text{PO}_4$ 0.2–0.5%, NaNO_3 0.2–0.5%, pH 5.0–7.0 (design-1); temperature 25–35 °C, incubation time 4.0–6.0 days, inoculum size 1.0–3.0 mycelial agar discs and agitation 100–200 rpm (design-2) were selected for the present investigation. The optimised medium variables (lactose 3.70%, $\text{NH}_4\text{H}_2\text{PO}_4$ 0.35%, NaNO_3 0.35% and pH 6.0) produced 44.44 (IU/ml) and 0.38 (g dry wt./50 ml) of inulinase and biomass yield, respectively. Thereafter, the optimization of process conditions (temperature 25 °C, incubation time 5 days, inoculum size 2 mycelial agar discs and agitation 150 rpm), increased the inulinase production 50.45 (IU/ml) and biomass yield 0.26 (g dry wt./50 ml). The good agreement between experimental and predicted values in both the designs, the coefficient of determination (R^2) greater than 0.90 and very close to 1.0 shows an appropriate fitness of the polynomial quadratic models.

Keywords *Penicillium oxalicum* · Inulinase · Lactose · Response surface methodology

Introduction

Inulin is a plant-derived storage polysaccharide which consists of (2 → 1) linked β -D-fructosyl residues and terminated with a (1 → 2) bonded α -D-glucose moiety. It is the second most abundant carbohydrate after starch (Franck 2002). *Helianthus tuberosus*, *Chicory intybus*, *Dahlia pinnata*, *Allium cepa*, *Asparagus* sp., etc. are some common inulin-rich plants (Singh et al. 2017). Inulin is used as a carbon source as well as an inducer for the production of inulinases, but it is an expensive substrate. Inulinases are inducible enzymes, which require an inducer (inulin) for their synthesis. Therefore, many researchers have made efforts to utilise other economical carbon sources such as sucrose, starch, fructose, etc. in combination with a small quantity of inulin (as an inducer) to produce inulinases from

various microorganisms (Singh and Chauhan 2016; Singh et al. 2017). Inulinases are reported from animals, plants and microorganisms. However, microbial sources are a preferred choice because of their easy cultivation, genetic alteration and high enzyme yield. Amongst different microbial sources, now the focus has been inclined towards the use of fungal sources due to their advantageous features such as wide thermal and pH stability. *Aspergillus* sp. and *Penicillium* sp. are the most explored fungal sources, due to their high inulinase yield (Singh and Chauhan 2016). Inulinases are mainly categorised into exo- and endo-inulinase. Exo-inulinase (EC 3.2.1.80) sequentially hydrolyses inulin from its terminal end to release fructose units, therefore, exclusively used for high-fructose syrup preparation (Singh et al. 2007a, b, 2008; Singh and Singh 2017). However, endo-inulinase (3.2.1.7) acts randomly on internal linkages of polymer to produce fructooligosaccharides (Singh and Singh 2010; Singh et al. 2016a, b).

In a previous study by our group, *P. oxalicum* BGPUP-4 isolated from soil from the vicinity of roots of inulin-rich plants has been reported as a new exo-inulinase producer (Singh and Chauhan 2017). Furthermore, various carbon sources were screened for the inulinase production from *P.*

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oxalicum BGPUP-4 and lactose has been proved as the best carbon source (Singh and Chauhan 2017). Medium formulation and process variables at an optimal level are the most fundamental aspects to obtain maximum enzyme yield. In this context, response surface methodology (RSM) is generally applied to study the simultaneous influence of numerous factors, and can be more beneficial. It also minimises the occurrence of error while determining the effect of each factor. Furthermore, it reduces the optimization time compared to tedious classical approach which involves the change of one-variable-at-a-time. The present study is an extension of our previous work, here a sequential approach using central composite rotatable design (CCRD) of RSM has been used to optimise the lactose-based medium and process variables for inulinase production from *Penicillium oxalicum* BGPUP-4 in shake-flask fermentations. Literature survey reveals that this is a first report on inulinase production from a lactose-based medium using *P. oxalicum* BGPUP-4.

Materials and methods

Fungal culture

Penicillium oxalicum BGPUP-4, an isolate of our laboratory was maintained on potato dextrose agar (PDA) slants as described previously (Singh and Chauhan 2017).

Inoculum preparation

Inoculum was prepared by growing an active *P. oxalicum* stock culture on PDA plates for 5 days at 30 °C. After incubation, 2 agar discs (10 mm diameter), uniformly covered with fungal mycelium were cut and used as an inoculum to inoculate the production medium (50 ml) in Erlenmeyer's flasks (250 ml).

Sequential statistical optimization of inulinase production from *P. oxalicum* BGPUP-4 in shake-flask fermentations

CCRD of RSM was used for the sequential optimization of a lactose-based medium (design-1) and process parameters (design-2) for inulinase production from *P. oxalicum* BGPUP-4. RSM is a collection of statistical tools which helps in generating an optimal relationship between response variables to produce best and optimised responses. In the present study, a 2⁴ factorial CCRD design was used to decipher optimal combinations of four independent variables (in each design) for inulinase production and biomass yield. Design expert version 7.0.0[®] (State-Ease Inc., Minneapolis, MN, USA), statistical software package was employed for the present analysis. The selected range of four independent variables (for design-1 and design-2) in terms of five coded values are given in Table 1. During medium optimization, other media constituents viz., inulin (0.5%), KH₂PO₄ (0.2%), MgSO₄·7H₂O (0.05%) and FeSO₄·7H₂O (0.001%) were kept constant and shake-flask fermentations were carried out at 30 °C for 5 days, under agitation (150 rpm). A total 21 trial combinations, for each design, with eight fractional, eight axial points and five replicates at the centre points were performed. Hence, actual sixteen combinations were resulted from each experimental design. Experimental runs 4, 7, 8, 17 and 20 (design-1) and 3, 5, 8, 11 and 14 (design-2) at the centre points were used to ascertain the replicability and inaccuracy in the experiments. Each experiment was performed in triplicates to remove the probability of any error.

A second-order polynomial equation used to analyse the experimental data is given below:

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum A_{ij} X_i X_j + e, \quad (1)$$

Table 1 Factors and values of coded levels chosen for experimental designs

Factors	Code	Actual levels of coded factors				
		− 1.68	− 1	0	1	1.68
<i>Design 1^a</i>						
Lactose (%)	A	0.32	1	2	3	3.7
NH ₄ H ₂ PO ₄ (%)	B	0.1	0.2	0.35	0.5	0.6
NaNO ₃ (%)	C	0.1	0.2	0.35	0.5	0.6
pH	D	4.3	5	6	7	7.7
<i>Design 2^b</i>						
Temperature (°C)	E	25.0	25	30	35	39.0
Incubation time (Days)	F	3.5	4	5	6	6.5
Inoculum size (Discs)	G	0.5	1	2	3	3.5
Agitation (rpm)	H	65.0	100	150	200	235.0

^aMedium optimization variables

^bProcess optimization variables

where, Y is the response, A_0 is constant coefficient, A_i , A_{ii} and A_{ij} are regression coefficients of the model, while X_i and X_j represents the independent variables and e is the error of the model.

Statistical significance of the data was tested using analysis of variance (ANOVA) for polynomial models with 95% confidence level. Fischer's F test and student's t test were also adopted to discover significance of second-order model equation and regression coefficients, respectively. Moreover, regression and three-dimensional (3D) surface analysis were performed to envision the effect of interactions between different variables on desired responses. Additionally, coefficient of determination (R^2), adjusted coefficient of determination (Adj. R^2) and adequate precision were used to determine the quality of fitted regression models.

Analytical techniques

Inulinase assay

Crude enzyme (0.1 ml) was mixed with 0.9 ml of 2% inulin solution (prepared in 0.1 M sodium acetate buffer, pH 5.0). Reaction mixture was incubated at 55 °C for 10 min. Then, dinitrosalicylic acid was added and resulting mixture was heated in a boiling water bath for 15 min. Thereafter, resulting mixture was examined for reducing sugars by dinitrosalicylic acid method (Miller 1959). One inulinase unit is defined as the amount of enzyme that produces one micromole of fructose per minute, under standard assay conditions.

Biomass yield determination

After shake-flask fermentations, the mycelial mass was filtered through a Whatman filter paper No. 1. and washed twice with distilled water. Fungal biomass was then dried in an oven at 50 °C, until a constant weight was obtained. Biomass yield is expressed as g dry wt./50 ml.

Results and discussion

Model development and analysis of variance (ANOVA)

RSM-designed experimental trials were performed to study the effect of independent variables on selected responses. The best model from linear, 2F1, quadratic and cubic models was determined using a multiple regression analysis of Design expert 7.0.0 statistical software. Quadratic model showed highest F value in comparison with the other models. Hence, it has been evaluated using sum of squares and analysis of variance. Tables 2 and 3 summarise the design

matrix for experimental and predicted results obtained after sequential statistical optimization of lactose-based medium (design-1) and process variables (design-2) for two responses, namely inulinase production and biomass yield. F value was used to statistically test the overall significance of the regression model, evaluate the specific regression terms and fitness of the model terms. The computed model F value of design-1 for inulinase production and biomass yield was 36378.62 and 1089.20, respectively. Whereas, for design-2, it was calculated as 1337.13 and 177.39 for inulinase production and biomass yield, respectively. Such higher F value suggested the significance of the model and 0.01% occurrence chance of this large value may be due to the noises (Tables 4, 5). The values of regression coefficients were calculated and the following second-order polynomial equations (in terms of coded values), regardless of significant terms were generated:

Design-1

$$\begin{aligned} \text{Inulinase production} = & +13.03 + 7.77 * A + 0.33 * B + 1.84 * C \\ & - 0.26 * D - 0.71 * A * B + 3.01 * A * C + 0.57 * A * D \\ & - 0.089 * B * C + 0.12 * B * D - 0.60 * C * D \\ & + 6.48 * A^2 - 3.44 * B^2 - 3.15 * C^2 - 2.54D^2 \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Biomass yield} = & +0.88 + 0.16 * A + 0.20 * B \\ & - 0.018 * C + 2.378E - 003 * D + 0.042 * A * B \\ & - 0.069 * A * C + 0.098 * A * D - 0.090 * B * C \\ & - 0.020 * B * D + 0.083 * C * D - 0.14 * A^2 \\ & - 0.085 * C^2 - 0.067D^2, \end{aligned} \quad (3)$$

where A , is lactose (%); B , $\text{NH}_4\text{H}_2\text{PO}_4$ (%); C , NaNO_3 (%) and D , pH.

Design-2

$$\begin{aligned} \text{Inulinase production} = & +26.50 - 8.4 * E + 2.18 * F \\ & + 1.26 * G - 3.39 * H - 5.23 * E * F + 1.14 * E * G \\ & + 7.04 * E * H + 2.13 * F * G - 6.52 * F * H \\ & - 2.44 * G * H + 3.44 * E^2 + 3.53 * F^2 \\ & - 1.25 * G^2 + 0.28 * H^2 \end{aligned} \quad (4)$$

$$\begin{aligned} \text{Biomass yield} = & +0.39 - 0.021 * E + 0.095 * F \\ & - 0.020 * G + 0.062 * H + 0.080 * E * F \\ & + 0.017 * E * G + 0.095 * E * H - 0.033 * F * G \\ & - 0.016 * F * H + 0.034 * E^2 + 0.018 * F^2 \\ & - 0.026 * G^2 + 0.094 * H^2, \end{aligned} \quad (5)$$

where E , is temperature (°C); F , incubation time (days); G , inoculum size (discs) and H , agitation (rpm).

Table 2 Central composite rotatable design (CCRD) matrix of medium components for inulinase production and biomass yield from *P. oxalicum* BGPUP-4

Runs	Factors ^a				Experimental results		Predicted values	
	A	B	C	D	Inulinase production (IU/ml)	Biomass yield (g dry wt./50 ml)	Inulinase production (IU/ml)	Biomass yield (g dry wt./50 ml)
1	3.0	0.2	0.2	7.0	14.34	0.27	14.38	0.27
2	2.0	0.35	0.35	4.3	6.29	0.35	6.28	0.35
3	2.0	0.1	0.35	6.0	2.76	0.28	2.75	0.28
4	2.0	0.35	0.35	6.0	12.98	0.44	13.03	0.44
5	2.0	0.35	0.60	6.0	7.16	0.30	7.22	0.30
6	3.7	0.35	0.35	6.0	44.44	0.38	44.43	0.38
7	2.0	0.35	0.35	6.0	13.06	0.44	13.03	0.44
8	2.0	0.35	0.35	6.0	13.02	0.44	13.03	0.44
9	2.0	0.6	0.35	6.0	3.87	0.61	3.86	0.61
10	1.0	0.5	0.5	7.0	1.11	0.26	1.09	0.26
11	0.32	0.35	0.35	6.0	18.31	0.11	18.3	0.11
12	1.0	0.2	0.2	5.0	2.96	0.14	3.0	0.14
13	2.0	0.35	0.35	7.7	5.43	0.35	5.42	0.35
14	2.0	0.35	0.1	6.0	1.1	0.34	1.02	0.33
15	3.0	0.2	0.5	7.0	23.08	0.36	23.06	0.36
16	3.0	0.5	0.5	5.0	22.72	0.33	22.7	0.33
17	2.0	0.35	0.35	6.0	13.07	0.44	13.03	0.44
18	1.0	0.2	0.5	5.0	2.06	0.20	2.04	0.20
19	1.0	0.5	0.2	7.0	4.8	0.21	4.81	0.22
20	2.0	0.35	0.35	6.0	13.0	0.44	13.03	0.44
21	3.0	0.5	0.2	5.0	11.93	0.59	11.97	0.59

^aSymbols A, B, C & D are same as indicated in Table 1

ANOVA justifies the statistical significance of Eqs. 2, 3, 4 and 5 for the quadratic polynomial model. The statistical data in Tables 4 and 5 substantiates the significance of each model. The Prob > *F* value less than 0.05 testify the significance of model terms, while value greater than 0.10 symbolises non-significant model terms. Hence, smaller Prob > *F* value and higher model *F* value shows significance of the resultant coefficient terms (Singh et al. 2009; Singh and Saini 2013; Singh and Yadav 2013; Singh et al. 2014; Singh et al. 2016a). The model terms with *P* > 0.0001 are considered as the most significant model terms. In the present study, A, B, C, D, AB, AC, AD, CD, A², B², C², D² and A, B, C, AB, AC, AD, BC, CD, A², C², D² are the most significant model terms for inulinase production and biomass yield, respectively, in design-1. For design-2, terms E, F, G, H, EF, EG, EH, FG, FH, GH, E², F², G², and F, G, EF, EH, E², G², H² are the most significant for inulinase production and biomass yield, respectively (Tables 4, 5). “Lack of Fit” sum of squares is one of the important element of ANOVA which exhibits the functional relationship between experimental factors and response variables. Non-significant “Lack of Fit *F* value” is good, which justifies the fitness of the model. A “Lack of Fit *F* value” 6.16 and 1.42 for design-1, and 0.78 and 0.83 for design-2 for inulinase

production and biomass yield, respectively, manifests the insignificant proportionality of Lack of Fit to the pure error and fitness of the model. If the design has repeated values or replicates, i.e., more than one run showing the same values for all model terms, then there will be a degree of freedom for pure error. Each repeated value contributes – 1 degree of freedom for pure error. Therefore, degree of freedom for pure error between 3 and 4 validates the significance of the model. In present investigation, the degree of freedom for pure error was found to be 4 for both the designs, which again validates the authenticity of the model. The goodness of fit of the model helps in depicting the good correlation between factor’s actual and predicted values. The coefficient of determination (*R*²) for inulinase production and biomass yield was calculated to be 1.0 and 0.9995 for design-1; whereas for design-2, its value was 0.9997 and 0.9970 for inulinase production and biomass yield, respectively. These values are very close to 1, which elucidates very minute percent variability in the responses. The adjusted *R*² and predicted *R*² for inulinase production after medium optimization were 1.0 and 0.9994; whereas for biomass yield, these values were 0.9986 and 0.9890, respectively. Besides, adjusted *R*² and predicted *R*² values for inulinase production after process variable optimization were 0.9989 and

Table 3 Central composite rotatable design (CCRD) matrix of process variables for inulinase production and biomass yield from *P. oxalicum* BGPUP-4

Runs	Factors ^a				Experimental results		Predicted values	
	<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>	Inulinase production (IU/ml)	Biomass yield (g dry wt./50 ml)	Inulinase production (IU/ml)	Biomass yield (g dry wt./50 ml)
1	39.0	5.0	2.0	150.0	22.18	0.23	22.09	0.23
2	30.0	3.5	2.0	150.0	32.92	0.14	32.83	0.14
3	30.0	5.0	2.0	150.0	25.98	0.19	26.50	0.19
4	25.0	6.0	1.0	200.0	31.48	0.28	31.55	0.28
5	30.0	5.0	2.0	150.0	26.58	0.20	26.50	0.19
6	30.0	5.0	2.0	65.0	33.08	0.28	32.99	0.28
7	30.0	5.0	3.5	150.0	25.19	0.14	25.10	0.14
8	30.0	5.0	2.0	150.0	26.59	0.20	26.50	0.19
9	25.0	6.0	3.0	200.0	31.13	0.22	31.19	0.22
10	35.0	6.0	3.0	100.0	30.83	0.24	30.89	0.24
11	30.0	5.0	2.0	150.0	26.52	0.19	26.50	0.19
12	35.0	4.0	1.0	200.0	39.42	0.23	39.49	0.23
13	25.0	5.0	2.0	150.0	50.45	0.26	50.36	0.26
14	30.0	5.0	2.0	150.0	26.6	0.19	26.50	0.19
15	30.0	5.0	2.0	235.0	21.69	0.38	21.60	0.38
16	30.0	6.5	2.0	150.0	40.25	0.30	40.16	0.30
17	35.0	4.0	3.0	200.0	35.09	0.26	35.15	0.26
18	25.0	4.0	3.0	100.0	37.77	0.26	37.83	0.26
19	30.0	5.0	0.5	150.0	20.94	0.18	20.85	0.18
20	25.0	4.0	1.0	100.0	36.91	0.27	36.98	0.27
21	35.0	6.0	1.0	100.0	16.89	0.28	16.96	0.28

^aSymbols *E*, *F*, *G* & *H* are same as indicated in Table 1

0.9955; while the biomass yields were 0.9914 and 0.9608, respectively. Difference less than 0.2 between adjusted R^2 and predicted R^2 showed their rational agreement with each other, which again validates the present models. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is anticipated. The higher adequate precision denotes efficiency of the models to navigate the design space. In the present model, adequate precision was 809.288 and 125.704 (design-1), and 153.882 and 55.744 (design-2) for inulinase production and biomass yield, respectively.

Effect of independent and interacting lactose-based medium variables on inulinase production

Three-dimensional (3D) surface graphs were used to study the effects of four independent variables on inulinase production (Fig. 1) and biomass yield (Fig. 2) from *P. oxalicum* BGPUP-4. The 3D graphs are helpful in understanding the effect of variables by pairwise combination, while holding the other two factors at mid-level. In the present study, lactose was used as a sole carbon source for inulinase production, while inulin concentration was kept constant throughout the study. It is evident from the plots that lactose concentration has a pronounced effect on

inulinase production. Inulinase production increased with the increase in lactose concentration. Maximum inulinase production (44.44 IU/ml) was observed at 3.70% lactose concentration. Low lactose concentration has shown a significant decrease in inulinase production in shake-flask fermentations. The increase in inulinase production in a lactose-based medium may be due to the change in cell wall structure of *P. oxalicum* BGPUP-4. Pessoni et al. (2015) reported the effect of different carbon sources on the cell wall of *Penicillium janczewskii*. They reported an increase in extracellular inulinase production in fructose-containing medium due to the change in cell wall structure of the microorganism. Hence, it can be inferred that carbon source plays an important role in enzyme production. Inulinases are inducible enzymes, which require inulin for their biosynthesis. However, inulin is a costly substrate; its diminutive amount in combination with a low-cost substrate can be a good alternative for inulinase production. Lactose itself cannot act as an inducer, but can be a persuasive carbon source for microbial growth. Proper microbial growth entails enhanced metabolite production, which approves our findings on a higher inulinase production from lactose in combination with very less inulin concentration (0.5%). Our findings are also in agreement

Table 4 Analysis of variance (ANOVA) and regression analysis of suitable quadratic model for the medium optimization

Source ^a	ANOVA				Regression analysis					
	Inulinase production (IU/ml)		Biomass yield (g dry wt./50 ml)		Inulinase production (IU/ml)		Biomass yield (g dry wt./50 ml)		F value	F value
	Sum of squares	df	Prob > F	Sum of squares	df	Prob > F	Coefficient estimate	Standard error		
Model/Intercept	2051.38	14	< 0.0001	0.33	13	< 0.0001	13.03	0.027	36378.62	0.44
A	341.39	1	< 0.0001	0.036	1	< 0.0001	7.77	0.027	84757.18	0.080
B	0.62	1	< 0.0001	0.054	1	< 0.0001	0.33	0.027	152.95	0.098
C	46.36	1	< 0.0001	1.186E-003	1	< 0.0001	1.84	0.017	11509.48	- 9.319E-003
D	0.37	1	< 0.0001	0.00	1	1.000	- 0.26	0.027	91.81	0.000
AB	1.67	1	< 0.0001	1.325E-003	1	< 0.0001	- 0.71	0.035	414.06	0.026
AC	72.54	1	< 0.0001	9.800E-003	1	< 0.0001	3.01	0.022	18009.90	- 0.035
AD	1.07	1	< 0.0001	6.893E-003	1	< 0.0001	0.57	0.035	266.13	0.046
BC	0.06	1	0.0075	0.016	1	< 0.0001	- 0.09	0.022	15.64	- 0.045
BD	0.05	1	0.0127	4.955E-004	1	0.0025	0.12	0.035	12.29	- 0.012
CD	2.89	1	< 0.0001	0.014	1	< 0.0001	- 0.60	0.022	718.01	0.043
A ²	627.63	1	< 0.0001	0.074	1	< 0.0001	6.48	0.016	1.558E+005	- 0.070
B ²	176.85	1	< 0.0001	-	-	-	- 3.44	0.016	43906.96	-
C ²	148.46	1	< 0.0001	0.029	1	< 0.0001	- 3.15	0.016	36859.59	- 0.044
D ²	96.43	1	< 0.0001	0.017	1	< 0.0001	- 2.54	0.016	23942.06	- 0.033
Residual	0.02	6		1.653E-004	7					
Lack of fit	0.018	2	0.0600	8.535E-004	3	0.3600			6.16	
Pure error	5.920E-003	4		8.000E-005	4					
Cor total	2051.41	20		0.33	20					

^aSymbols A, B, C & D are same as indicated in Table 1

Table 5 Analysis of variance (ANOVA) and regression analysis of fitted quadratic model for process variables optimization

Source ^a	ANOVA				Regression analysis					
	Inulinase production (IU/ml)		Biomass yield (g dry wt./50 ml)		Inulinase production (IU/ml)		Biomass yield (g dry wt./50 ml)			
	Sum of squares	df	Prob > F	Sum of squares	df	Prob > F	Coefficient estimate	Standard error	F value	F value
Model/Intercept	1234.83	14	< 0.0001	0.064	13	< 0.0001	26.50	0.11	1337.13	0.19
<i>E</i>	399.60	1	< 0.0001	4.500E-003	1	0.0050	− 8.40	0.11	6057.82	− 8.919E-003
<i>F</i>	26.86	1	< 0.0001	0.013	1	< 0.0001	2.18	0.11	407.26	0.048
<i>G</i>	21.83	1	< 0.0001	1.58E-003	1	0.0001	1.26	0.069	330.99	− 0.011
<i>H</i>	64.87	1	< 0.0001	5.00E-003	1	< 0.0001	− 3.39	0.11	983.36	0.030
<i>EF</i>	90.51	1	< 0.0001	4.593E-003	1	< 0.0001	− 5.23	0.14	1372.12	0.037
<i>EG</i>	10.35	1	< 0.0001	4.500E-003	1	0.0050	1.14	0.091	156.92	7.500E-003
<i>EH</i>	164.08	1	< 0.0001	7.498E-003	1	< 0.0001	7.04	0.14	2487.43	0.048
<i>FG</i>	36.38	1	< 0.0001	1.800E-003	1	< 0.0001	2.13	0.091	551.52	− 0.015
<i>FH</i>	140.96	1	< 0.0001	1.365E-004	1	0.06272	− 6.52	0.14	2136.98	− 6.419E-003
<i>GH</i>	47.43	1	< 0.0001	−	−	−	− 2.44	0.091	719.09	−
<i>E</i> ²	176.70	1	< 0.0001	4.586E-003	1	< 0.0001	3.44	0.066	2678.69	0.018
<i>F</i> ²	186.64	1	< 0.0001	1.126E-003	1	0.0004	3.53	0.066	2829.48	8.680E-003
<i>G</i> ²	23.20	1	< 0.0001	2.348E-003	1	< 0.0001	− 1.25	0.066	351.72	− 0.013
<i>H</i> ²	1.18	1	0.0055	0.034	1	< 0.0001	0.28	0.066	17.93	0.048
Residual	0.40	6		1.946E-004	7					
Lack of fit	0.11	2	0.5175	7.463E-005	3	0.5430			0.78	
Pure error	0.28	4		1.200E-004	4					
Cor total	1235.22	20		0.064	20					

^aSymbols *E*, *F*, *G* & *H* are same as indicated in Table 1

Fig. 1 Response surface 3D plots showing the effect of interaction between: **a** $\text{NH}_4\text{H}_2\text{PO}_4$ and NaNO_3 , **b** $\text{NH}_4\text{H}_2\text{PO}_4$ and pH, **c** NaNO_3 and pH, and **d** lactose and pH on inulinase production from *P. oxalicum* BGPUP-4

with inulinase production from *Bacillus polymyxa*, *B. subtilis* (Zherebtsov et al. 2002) and *Aspergillus niger* (Al-Dabbagh et al. 2015), where inulinase synthesis has been reported in starch- and sucrose-supplemented production medium, respectively. Nitrogen sources are also one of the key factors for obtaining good enzyme yield. Nitrogen is an important component of various amino acids, precursors and nucleic acids, which are major prerequisites for directing pertinent microbial growth as well as metabolites produced by microorganisms. Generally, fungi require higher concentration of nitrogen sources for the production of good amount of desirable metabolites (Iwai and Tsujisaka 1984). The different biochemical events which contribute to fungal sporulation are yet not well

known. Accordingly, the best way to observe peculiar-related changes in fungal sources is to carry out the optimisation study of medium constituents, which alters these events (Singh 1971). In present study, $\text{NH}_4\text{H}_2\text{PO}_4$ (0.35%) and NaNO_3 (0.35%) supported maximum inulinase production (Fig. 1a). At very low and high concentration of these nitrogen sources, a reduction in inulinase production was observed. This may be imputed to the suppression of biochemical processes at very low and high concentration of nitrogen, which resulted in stunted fungal hyphal growth and further inhibiting the inulinase biosynthesis. Gavrilov et al. (2016) observed rapid cellular growth of *Bacillus* sp. in the presence of 0.4% peptone, beef extract, yeast extract, casein and $(\text{NH}_4)_2\text{SO}_4$. The combination of

Fig. 2 Response surface 3D plots showing the effect of interaction between: **a** lactose and NaNO_3 , **b** lactose and pH, **c** $\text{NH}_4\text{H}_2\text{PO}_4$ and pH, and **d** NaNO_3 and pH on biomass yield from *P. oxalicum* BGPUP-4

these nitrogen sources with inulin-rich plant material supported 6–10 times more inulinase production. Hence, use of nitrogenous surrounding at certain level can be facilitative in increasing the inulinase production. The production medium pH also had a prominent effect on enzyme production. Maximum inulinase production was obtained at pH 6.0. Any further change in pH caused a reduction in enzyme yield (Fig. 1b, c). This may be ascribed to alterations in cell's surface caused by change in the pH of the medium, which consequently interrupts metabolite production. Our study corroborates the findings on inulinase production in acidic environment from *A. oryzae* (Ali and Shahzadi 2015), *A. tritici* (Singh et al. 2016a), *A. tubingenensis* (Trivedi et al. 2012) and *P. funiculosum* (Danial and Al-Bishri 2015).

The maximum biomass yield (0.61 g dry wt./50 ml) was obtained at mid-values of four variables viz., lactose, 2%; $\text{NH}_4\text{H}_2\text{PO}_4$, 0.6%; NaNO_3 , 0.35% and pH 6.0, while the biomass yield during maximum inulinase producing run 6 was 0.38 g dry wt./50 ml. Hence, it can be inferred from the observation that increase in biomass is not always proportional to enzyme production. Increase in fungal biomass mainly depends upon the type of media, moisture availability and temperature. However, enzyme production increases with the increase in biomass up to a certain level, later on changes in the microenvironment with respect to time may produce other secondary metabolites, which may bring conformational deviances in the enzyme structure. Our results corroborate the same pattern for biomass yield and inulinase production from *B. safensis* (Singh et al. 2014) and *A. tritici* (Singh et al. 2016a).

The interactive effect of lactose, $\text{NH}_4\text{H}_2\text{PO}_4$, NaNO_3 and pH on inulinase production and biomass yield was also investigated. It was pragmatic that lactose (3.70%) and both the nitrogen sources (0.35%) supported maximum inulinase production. The synergism between $\text{NH}_4\text{H}_2\text{PO}_4$ and NaNO_3 displayed a positive effect on inulinase production and biomass yield, but up to a certain level. A very high and low concentration of these constituents retarded the inulinase production (Fig. 1a). Lactose proved to be a best carbon source for inulinase production from *P. oxalicum* BGPUP-4. Its higher concentration along with mid-range of nitrogen sources supported maximum inulinase production. However, at very low lactose (1%) and very high $\text{NH}_4\text{H}_2\text{PO}_4$ (0.50%) and NaNO_3 (0.50%) concentration, inulinase production reduced from 44.44 to 1.11 IU/ml. Usually, nitrogen sources easily dissociate into ions which subsequently alter pH of the medium, while sugars impose no such type of changes. No sudden negative and positive effect of lactose on inulinase production and biomass yield during its interactions with pH was observed. However, $\text{NH}_4\text{H}_2\text{PO}_4$ and NaNO_3 interaction

with pH demonstrated a significant effect on inulinase production (Fig. 1b, c) and biomass yield (Fig. 2c, d). This may be accredited to the contribution of ammonium, sodium and nitrate ions on sustaining pH of the medium, which perceptibly affected the inulinase production and biomass yield.

Effect of independent and interacting process variables on inulinase production

After medium optimization, the interactive effect of process variables (temperature, incubation time, inoculum size and agitation) on inulinase production and biomass yield was also studied. 3D surface curves against two independent variables, while keeping the third variable at its central level were analysed (Figs. 3, 4). The response surface graphs demonstrate the independent, interactive and synergistic effect of process parameters. The evaluation of 3D plots describes the effect of temperature on inulinase production (Fig. 3a). The maximum inulinase production (50.45 IU/ml) was obtained at temperature 25 °C and further increase in temperature

Fig. 3 Response surface 3D plots depicting the effect of: **a** temperature and inoculum size, **b** incubation time and agitation, and **c** agitation and inoculum size on inulinase production from *P. oxalicum* BGPUP-4

Fig. 4 Response surface 3D plots depicting the effect of: **a** temperature and incubation time, and **b** temperature and inoculum size on biomass yield from *P. oxalicum* BGPUP-4

reduced the inulinase biosynthesis. A temperature range 25–30 °C was found most suitable for inulinase production from *P. oxalicum* BGPUP-4, whereas at very high temperature (39 °C), a very less inulinase production was recorded. A decrease in oxygen solubility with increase in temperature can be attributed to retarded inulinase production. Moreover, high temperature creates physical changes in microbial cell membrane which further influences secretion and structure of extracellular enzyme (Dinarvand et al. 2013). The optimal temperature for growth and metabolite production for most of the fungal sources lies between 25–30 °C. In a number of studies, high inulinase production at low temperature from *A. niger* (Dinarvand et al. 2017), *A. tamarii* (Saber and Noura 2009), *P. subrubescens* (Mansouri et al. 2013) and *Rhizopus oligosporus* (Mohamed et al. 2015) has also been reported. Incubation time and inoculum size are also very crucial factors for enzyme production. Maximum inulinase production (50.45 IU/ml) was observed, when medium was inoculated with two mycelial agar discs and shake-flask fermentation was carried out for 5 days. Incubation period above 5 days showed a decline in inulinase production, which maybe occurred either due to depletion of growth regulating the constituents in the medium or catabolite repression of the enzyme. Additionally, with the increase in incubation period, secretion of proteases into the medium takes place, which denatures the enzyme by disrupting its tertiary/quaternary structure as well as acidic shift in medium pH also occurs which lowers the enzyme production. Inoculum size less than two mycelial agar discs was not able to produce the sufficient biomass, correspondingly the desired inulinase yield as well. Inoculum size more than two mycelial agar discs also produced the low

enzyme yield. This may be attributed to the elevation in medium's viscosity with increased fungal biomass, creating a nutritional imbalance in the medium and making the cells physiologically incapable to produce the enzyme. Our findings corroborate the results of inulinase production from *Kluyveromyces marxianus* (Singh et al. 2007c) and *A. niger* (Dinarvand et al. 2017). Appropriate agitation rate is a basic requirement for carrying out successful fungal aerobic fermentations. Agitation not only supplies oxygen to the growing cells by increasing interface between gas and liquid, but also helps to eliminate waste gaseous products. However, high agitation rate can cause hyphal disruption and leakage of intracellular compounds by creating hydrodynamic stress (Bakri et al. 2011). In the present study, maximum inulinase production was obtained at 150 rpm. Agitation rate above and below 150 rpm has shown a decline in inulinase production. This can be ascribed to inflicted shear stress on fungal hyphae causing rupturing of cell wall and change in fungal morphology due to high agitation rate, while low agitation rate might not be sufficient to uniformly mix the nutrients and growing cells, leading to low inulinase production.

The process parameters also have a great impact on biomass yield from microbial sources. Maximum biomass yield (0.38 g dry wt./50 ml) was obtained from experimental run 15, while the biomass yield from maximum inulinase producing experimental run 13 was 0.26 (g dry wt./50 ml). Growth of microbial biomass solely depends upon nutrient availability, favourable surroundings and subtle agitation rate. Temperature 30 °C, incubation time 5 days, inoculum size two mycelial agar discs and agitation 235 rpm were found optimal for maximum biomass yield. Response surface studies on *B. safensis* (Singh et al. 2014) and *A. tritici*

(Singh et al. 2016a) also equate with our findings on biomass yield from *P. oxalicum* BGPUP-4.

The interactive effect of four independent variables at optimal level, i.e., temperature 25 °C, incubation time 5 days, inoculum size 2 mycelial agar discs and agitation 150 rpm supported maximum inulinase production (50.45 IU/ml) and the corresponding biomass yield was 0.26 (g dry wt./50 ml). 3D surface graph (Fig. 3c) shows an interactive effect of inoculum size and agitation on inulinase production. Accurate agitation rate helps in proper dissipation of heat and maintaining temperature of the fermentation medium. Although, agitation rate merely depends upon the viscosity of the medium, while inoculum size is the pivotal factor for maintaining viscosity of the medium. High inoculum size increases medium's viscosity with time due to excessive hyphal growth, which further obstructs heat dissipation rate by decreasing fluidity of the medium. This whole network of processes affects cell's physiognomy and subsequently enzyme production. Hence, it can be evidenced that interdependency of all factors at an optimal level shows an enhanced inulinase production (Fig. 3a, b, c) and biomass yield (Fig. 4a, b).

Validation of experimental model

To assess the proficiency of the model and to compare predicted and experimental results, the experimental trial of optimised conditions was conducted in triplicates. From design-1, with optimised conditions (lactose 3.70%, $\text{NH}_4\text{H}_2\text{PO}_4$ 0.35%, NaNO_3 0.35% and pH 6.0), the maximum inulinase production and the corresponding biomass yield obtained was 44.44 (IU/ml) and 0.38 (g dry wt./50 ml), respectively. The predicted value of inulinase production and biomass yield was 44.43 (IU/ml) and 0.38 (g dry wt./50 ml), respectively. The predicted values from the regression model closely agreed with the actual values, which authenticates the validity of the model and presence of an optimal point.

Experimental trials for the validation of design-2 were also performed in triplicates. The maximum experimental inulinase production and the corresponding biomass yield obtained from optimised process variables, i.e., temperature 25 °C, incubation time 5 days, inoculum size 2 mycelial agar discs and agitation 150 rpm, was 50.45 (IU/ml) and 0.26 (g dry wt./50 ml), respectively, while the values predicted by the model for inulinase production and biomass yield was 50.36 (IU/ml) and 0.26 (g dry wt./50 ml), respectively. The predicted values by the polynomial model and experimental values are in good agreement with each other, which again indicates model's validity.

Conclusions

The present study demonstrated the applicability of statistical optimization as an effective methodology for the optimization of significant variables for inulinase production. RSM not only provides the minimal required optimal level of utmost important factors, but also proved very useful in developing time-economic optimising process. After statistical optimization, a significant 1.31-fold increase in inulinase production was observed in shake-flask fermentations. Besides, on the basis of ANOVA, the quadratic polynomial model was deduced significant in exhibiting actual relationship between the response variables. Furthermore, R^2 value very close to one illustrated the significance of the quadratic model for envisaging inulinase production from *P. oxalicum* BGPUP-4. Moreover, it also elaborated the advantages of RSM in forecasting the effect of optimised conditions on desired responses. The medium optimization study has developed a cost-effective lactose-based medium for inulinase production from *P. oxalicum* BGPUP-4. *P. oxalicum* would be a good candidate for the production of inulinase from a lactose-based substrate (whey) in scale-up studies.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest in the publication of this manuscript.

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