

Enhancement of Inulinase and Invertase Production from a Newly Isolated *Candida guilliermondii* TISTR 5844

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ABSTRACT

The optimization of inulinase and invertase production from *Candida guilliermondii* TISTR 5844 was studied using a central composite design with four factors: pH, temperature, inoculum and substrate concentrations. The most significant factors influencing invertase production were pH and temperature with an R^2 value of 0.822 ($P < 0.05$). Unexpectedly, the R^2 value for inulinase production was 0.555 ($P > 0.05$) and there was no significant influence of the tested factors over the production of inulinase. The results obtained from the experimental design using one factor at a time demonstrated the optimum carbon and nitrogen sources for both enzymes were inulin and NH_4Cl , respectively. Optimization by Taguchi methodology further indicated the optimum medium compositions for the production of inulinase (inulin concentration 1%, NH_4Cl concentration 2.4%, $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ concentration 1.2% and pH 5) and invertase (inulin concentration 5%, NH_4Cl concentration 4.8%, $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ concentration 1.2% and pH 6).

Keywords: inulinase, invertase, *Candida guilliermondii*, optimization, response surface methodology, Taguchi method

INTRODUCTION

Inulinase (β -fructan fructanohydrolase) hydrolyzes inulin (a polysaccharide of β -(2,1)-linked fructose residues attached to a terminal glucose molecule) to fructose and fructo-oligosaccharides, both of which are important ingredients in the food and pharmaceutical industries (Vandamme and Derycke, 1983). Invertase (β -fructofuranosidase) breaks down sucrose to fructose and glucose by catalyzing the

hydrolysis of terminal nonreducing β -fructofuranoside residues in β -fructofuranosides. It is mainly used in the confectionery industry where fructose is preferred over sucrose because it is sweeter and does not easily crystallize (Rubio and Navarro, 2006). Inulinases are encountered in plants and many microorganisms whereas invertase is usually synthesized by yeast and filamentous fungi. *Candida guilliermondii* TISTR 5844, a novel effective inulinase and invertase producer, was isolated from Jerusalem artichoke

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tubers by Sirisansaneeyakul *et al.* (2007). This strain showed the ability to produce significant quantities of inulinase and invertase when inulin was used as the substrate, indicating its potential for industrial-scale utilization.

Since many enzymes of industrial significance are regulated by the medium composition and fermentation conditions, the study of this regulation is important in the commercial production of such enzymes. Optimization of the fermentation conditions and the medium composition to achieve a suitable balance is very important to obtain the optimum microbial growth and enzyme yield. Optimization of one factor at a time involves changing the independent variables while fixing the others at certain levels, to understand the impact of that particular parameter. However, this single dimensional (one factor at a time) search is laborious and time-consuming, especially for a large number of variables. Thus, response surface methodology (RSM) and the Taguchi method are preferred as they reduce the total number of experiments and take into account the interactions among the factors (Revankar and Lele, 2006). Therefore, the main objective of the present work was the improvement of the inulinase- and invertase-producing ability of *C. guilliermondii* TISTR 5844 by optimizing the fermentation conditions and the medium composition using a combination of RSM, the one factor at a time method and Taguchi methodology.

MATERIALS AND METHODS

Candida guilliermondii TISTR 5844 was obtained using the procedure in Sirisansaneeyakul *et al.* (2007) and used in this study. The inulinase and invertase activity were quantitatively determined according to Sirisansaneeyakul *et al.* (2007). One unit of inulinase activity was defined as the quantity liberating 1 μmol of fructose in 1 min from a 0.5% w/v inulin standard solution in

0.5 M McIlvaine buffer at pH 5.0 and 40 °C. One unit of invertase activity corresponded to the amount of enzyme liberating 1 μmol of fructose in 1 min from a 0.5% w/v sucrose solution in 0.5 M McIlvaine buffer at pH 5.0 and 40 °C.

Optimization of inulinase and invertase fermentation condition by RSM

All treatment combinations (Table 1) were performed in 500 mL Erlenmeyer flasks containing 200 mL of medium (1.2% yeast extract, 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 0.1 M McIlvaine buffer at pH 5). The experiments were performed and prepared according to the central composite design (CCD; Table 2). After 48 h of incubation, each flask was assayed for enzyme activity. RSM was used for the investigation of the effects of independent variables: pH, temperature, % inoculum and % substrate on the responses of inulinase and invertase activity. Using a CCD for four factors ($k = 4$), 30 treatment combinations were generated. To set up a statistical model, five levels for each variable were chosen. The upper and lower limits of each variable were chosen to encompass the range reported in the literature and to reflect what was done in practice after a preliminary investigation of the limits. The codes of $\pm\alpha(\pm 2)$ was designed at a distance of 2 ($2^{n/4} = 2$ for $n = 4$) from the design center. The remaining levels were identified using CCD (Moyo *et al.*, 2003). Table 1 shows the treatment combinations and responses. From the experimental data according to this design, a second order polynomial regression model was used (Equation 1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + \epsilon \quad (1)$$

where Y = inulinase or invertase production (U L^{-1}), b_i = the linear coefficients, b_{ii} = the quadratic coefficient, b_{ij} = the cross product coefficients and ϵ = the model constant.

Table 1 Treatment combinations and mean responses in the study.

Treatment	Actual factor level ^a				Inulinase activity (U L ⁻¹)	Invertase activity (U L ⁻¹)
	pH	Temperature	%	%		
			inoculum	inulin		
1	4.5	25	4.0	1.0	0.00	0.00
2	4.5	25	4.0	2.0	0.00	0.00
3	4.5	25	8.0	1.0	142.97	1267.83
4	4.5	25	8.0	2.0	1759.73	769.28
5	4.5	35	4.0	1.0	0.00	0.00
6	4.5	35	4.0	2.0	29.03	0.00
7	4.5	35	8.0	1.0	174.63	0.00
8	4.5	35	8.0	2.0	189.17	0.00
9	6.5	25	4.0	1.0	113.20	2313.75
10	6.5	25	4.0	2.0	161.58	3197.34
11	6.5	25	8.0	1.0	187.65	1910.50
12	6.5	25	8.0	2.0	202.16	2346.16
13	6.5	35	4.0	1.0	0.00	491.11
14	6.5	35	4.0	2.0	0.00	154.87
15	6.5	35	8.0	1.0	0.00	180.57
16	6.5	35	8.0	2.0	2.98	496.00
17	2.0	30	6.0	1.5	144.06	9.31
18	8.0	30	6.0	1.5	141.47	1121.15
19	5.0	20	6.0	1.5	242.74	1639.48
20	5.0	40	6.0	1.5	40.21	0.00
21	5.0	30	2.0	1.5	62.91	1226.13
22	5.0	30	10.0	1.5	145.21	114.33
23	5.0	30	6.0	0.5	212.61	921.91
24	5.0	30	6.0	2.5	212.98	635.23
25	5.0	30	6.0	1.5	115.40	891.42
26	5.0	30	6.0	1.5	125.48	929.75
27	5.0	30	6.0	1.5	149.69	722.40
28	5.0	30	6.0	1.5	148.28	621.72
29	5.0	30	6.0	1.5	146.86	759.68
30	5.0	30	6.0	1.5	169.43	627.42

^a = Levels based on the central composite design.

Optimization of inulinase and invertase fermentation medium by one factor at a time method and Taguchi methodology

One factor at a time method

To investigate the effect of the carbon source on inulinase and invertase production by

C. guilliermondii TISTR 5844, inulin was supplemented with glucose, fructose, sucrose and starch. To find out the effect of the nitrogen source, the yeast extract was replaced with different nitrogen sources such as beef extract, peptone, NH₄Cl, (NH₄)₂SO₄ and NH₄NO₃.

Taguchi methodology (Roy, 2001)

The Taguchi method was used to investigate the relationships between the components varied in the medium and to optimize their concentrations for inulinase and invertase production by *C. guilliermondii* TISTR 5844. Three levels of factor variation were considered and the size of experimentation was represented by the symbolic arrays L9. The symbolic designation of these arrays indicates the primary information on the size of the experiment, for example, L9 has nine trials. Each column consists of a number of conditions depending on the levels assigned to each factor. Four variables with three concentration levels were used. The variables optimized involved concentrations of inulin, NH_4Cl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and pH. The experimental set is shown in Table 2. Submerged fermentation experiments were carried out in 500 mL Erlenmeyer flasks containing 200 mL medium (1.2% yeast extract; inulin, 1, 3 and 5%; NH_4Cl ,

1.2, 2.4 and 4.8% and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2, 0.6 and 1.2%) in 0.1 M McIlvaine buffer, pH 4, 5 and 6). After 48 h of incubation, each flask was assayed for the activity of the different enzymes.

RESULTS AND DISCUSSION**Optimization of inulinase and invertase condition by Response Surface Methodology**

Data were analyzed using SPSS version 12 software for Windows (SPSS Inc., Chicago, IL, USA) to yield regression equations (Equations 2 and 3), regression coefficients and analysis of variance. From the analysis of variance (ANOVA), the invertase model was highly significant ($P < 0.05$; Table 3) with the R^2 value (indicating a measure of the goodness of fit of the model) showing that 82.20% of the total variation was explained by the model. The coefficient estimates in the regression model for invertase and inulinase are presented in Tables 4 and 5, respectively.

Table 2 L9 (3^4) orthogonal array design for production of enzymes by *C. guilliermondii* TISTR 5844.

Run	L9 (3^4)				Level of variables			Results (U L^{-1})		
	A	B	C	D	Inulin (%)	NH_4Cl (%)	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (%)	pH	Inulinase	Invertase
1	1	1	1	1	1	1.2	0.2	4	1.28	173.15
2	1	2	2	2	1	2.4	0.6	5	39.08	327.66
3	1	3	3	3	1	4.8	1.2	6	135.42	440.47
4	2	1	2	3	3	1.2	0.6	6	0.00	209.01
5	2	2	3	1	3	2.4	1.2	4	27.00	125.45
6	2	3	1	2	3	4.8	0.2	5	0.00	224.70
7	3	1	3	2	5	1.2	1.2	5	55.05	427.84
8	3	2	1	3	5	2.4	0.2	6	0.00	430.67
9	3	3	2	1	5	4.8	0.6	4	0.00	838.20

Table 3 Analysis of variance for evaluation of the second-order model for the model.

Source of variation	d.f.	Sum of squares	Mean square	F ratio	P-value
Model	14	16066534.0	1147609.589	4.932	0.002
Residual	15	3490493.7	232699.581		
Total	29	19557028.0			

d.f. = Degrees of freedom; $R^2 = 0.822$; Adjusted $R^2 = 0.555$.

Table 4 Estimated regression coefficients for invertase activity.

Term	Parameter estimate	P-value
Constant	-12342.773	0.101
pH	2818.864	0.010
Temperature	345.953	0.231
Inoculum	576.052	0.339
Substrate concentration	-590.037	0.804
pH × Temperature	-81.48	0.003
pH × Inoculum	-68.775	0.254
pH × Substrate concentration	244.780	0.308
Temperature × Inoculum	-4.701	0.702
Temperature × Substrate concentration	-21.038	0.669
Inoculum × Substrate concentration	-18.426	0.881
pH × pH	2.517	0.353
Temperature × Temperature	0.562	0.883
Inoculum × Inoculum	-5.831	0.807
Substrate concentration × Substrate concentration	15.036	0.969

Table 5 Estimated regression coefficients for inulinase activity.

Term	Parameter estimate	P-value
Constant	-3846.966	0.377
pH	332.483	0.572
Temperature	52.235	0.757
Inoculum	506.987	0.167
Substrate concentration	1308.339	0.363
pH × Temperature	7.170	0.612
pH × Inoculum	-51.397	0.158
pH × Substrate concentration	-150.808	0.293
Temperature × Inoculum	-10.500	0.166
Temperature × Substrate concentration	-40.827	0.177
Inoculum × Substrate concentration	98.211	0.193
pH × pH	-5.331	0.835
Temperature × Temperature	0.205	0.928
Inoculum × Inoculum	-1.058	0.941
Substrate concentration × Substrate concentration	91.814	0.688

However, the R^2 value of inulinase was 0.555 ($P > 0.05$) and indicated there was no significant influence of the studied factors on the inulinase production, and thus it could not be used to explain the factor relationships. Thus, the optimum condition was obtained from the regression model (Table 6).

After the treatment combinations, all linear terms of the independent variables, the quadratic term of pH and the interaction terms of pH with temperature (X_1X_2) were included in the model for invertase production since these were significant ($P < 0.05$). Thus, in the enzyme activity of *C. guilliermondii* TISTR 5844, the pH and

Table 6 Result of factor levels for the production of enzymes at optimum conditions.

Enzyme activity (U L ⁻¹)	pH	Temperature (°C)	Inoculum (%)	Substrate concentration (%)
Inulinase	2–6	30–40	6–10	> 3
Invertase	5–8	35–40	6–10	> 3

temperature were important, and therefore treating them together might reflect their true influence to the response, even though the quadratic terms of temperature, inoculum and substrate concentration were not found to be statistically significant ($P > 0.05$). The model equations for inulinase and invertase activities with the coefficients in coded units of factors are given in Equations 2 and 3, respectively:

$$\begin{aligned} \text{Inulinase production} = & 332.483X_1 + 52.235X_2 + \\ & 506.987X_3 + 1308.339X_4 + \\ & 7.170X_1X_2 - 51.397X_1X_3 - \\ & 150.808X_1X_4 - 10.50X_2X_3 - \\ & 40.827X_2X_4 + 98.211X_3X_4 - \\ & 5.331X_1^2 + 0.205X_2^2 - \\ & 1.058X_3^2 + 91.814X_4^2 - \\ & 3846.966 \quad (R^2=0.555) \quad (2) \end{aligned}$$

$$\begin{aligned} \text{Invertase production} = & 2818.864X_1 + 345.953X_2 + \\ & 576.052X_3 - 590.037X_4 - \\ & 81.486X_1X_2 - 68.775X_1X_3 + \\ & 244.780X_1X_4 - 4.701X_2X_3 - \\ & 21.038X_2X_4 - 18.426X_3X_4 + \\ & 2.517X_1^2 + 0.562X_2^2 - \\ & 5.831X_3^2 + 15.036X_4^2 - \\ & 12342.773 \quad (R^2=0.822) \quad (3) \end{aligned}$$

The effect of the interaction of various parameters on the inulinase and invertase production was studied by plotting three dimensional response curves against any two independent variables while keeping the other independent variable at their '0' levels. In predicting the response, all three-dimensional response surface graphs were generated using STATISTICA for Windows (Release 5.0, Statsoft, USA). Figure 1(a) depicts the three dimensional curve of the calculated response surface from the

interaction between pH, temperature, inoculum and substrate concentration while keeping pH and temperature at the '0' level. The whole surface is divided into 10 regions that present the response value range from 173.94 to more than 3958.491 U L⁻¹. Each region is identified using its lower range value. It can be concluded that the response surface plot indicates an optimum inulinase activity around a substrate concentration > 3% and 6–10% inoculum. When the pH and inoculum were fixed at 5 and 6% ('0' level), respectively, the maximum inulinase activity was obtained at a temperature of 30–40 °C with the substrate concentration > 3% (Figure 1(b)). Figure 1(c) shows the interaction of pH and substrate concentration at 6% inoculum and 30 °C ('0' level), and indicates that the maximum inulinase activity was achieved around pH 2–6 and a substrate concentration 6–10%. The substrate concentration and inoculum were fixed at 1.5 and 6%, respectively. The maximum inulinase activity was around pH 2–6 and a temperature of 20–30 °C (Figure 1(d)). Figures 1(e) and 1(f) show the interaction of temperature and pH with the substrate concentration at pH 5 and temperature 30 °C and 1.5% inulin ('0' level), indicating that the maximum inulinase activity was achieved around a temperature of 20–30 °C, pH 2–6 and 6–10% inoculum. Figures 2(a), 2(b) and 2(c) depict the three dimensional curves of the calculated response surface of the maximum invertase activity while keeping inoculum, temperature and substrate concentration at 6%, 30 °C and 1.5% inulin, respectively. The response surface plot indicated an optimum invertase activity around a temperature of 25–35 °C, pH 5–8, 6–10% inoculum and the substrate concentration

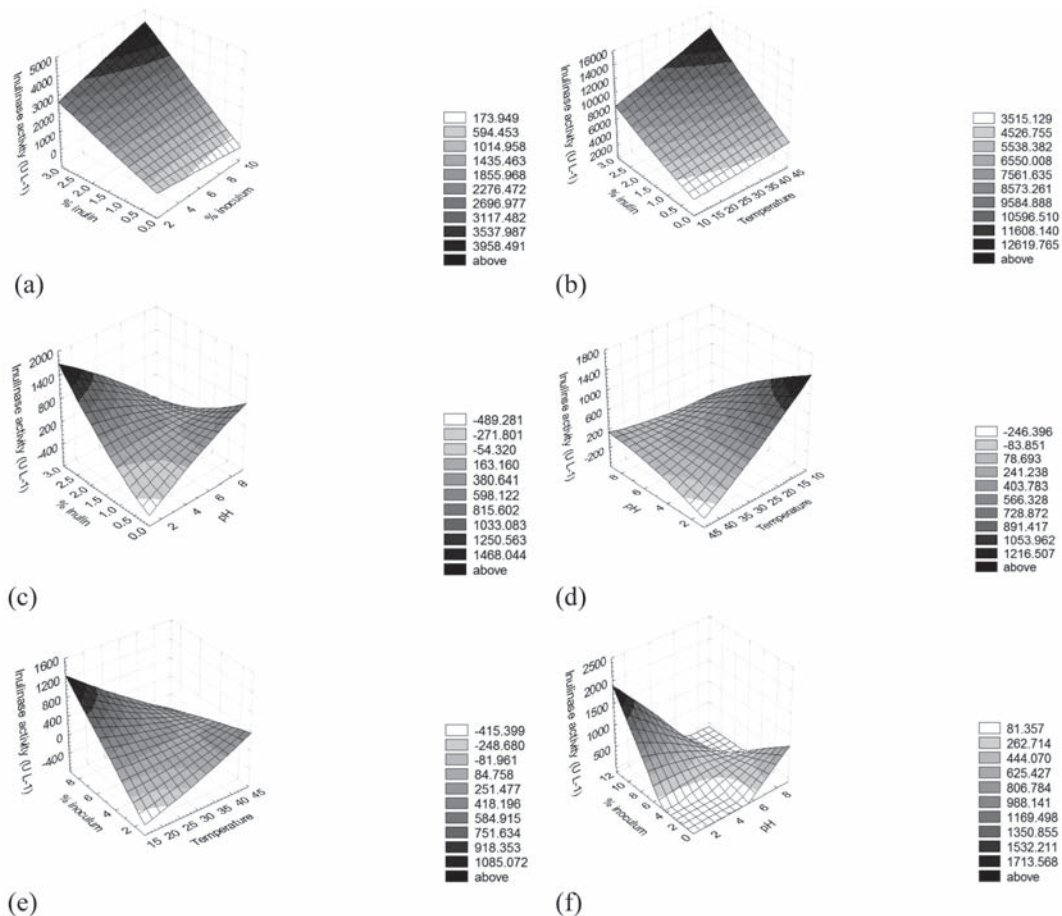


Figure 1 Response surface for the effect of factor on the inulinase activity after 48 h of incubations: (a) inoculum and substrate concentration at pH 5 and 30 °C; (b) temperature and substrate concentration at pH 5 and 6% inoculum; (c) pH and substrate concentration at 6% inoculum and 30 °C; (d) temperature and pH at 6% inoculum and 1.5% inulin; (e) temperature and inoculum at pH 5 and 1.5% inulin; and (f) pH and inoculum at 30 °C and 1.5% inulin.

> 3%. When the pH, inoculum, temperature and inulin concentration were fixed at 5, 6%, 30 °C and 1.5%, respectively, the maximum invertase activity was obtained at 6–10% inoculum, a temperature of 25–35 °C and inulin concentration > 3%. Taken all together, high inulinase and invertase activities were achievable. Similarly, the effect of the process parameters on inulinase production was studied by one factor at a time and fractional design. The optimal process conditions achieved a maximum inulinase activity at 5%

inoculum, temperature 28 °C, pH 6.5 with inulin being the best carbon source for enzyme production from *Kluyveromyces marxianus* YS-1 (Singh *et al.*, 2007). In another study, Wei *et al.* (1998) reported that maximum inulinase activity was obtained from *Kluyveromyces* sp.Y-85 at 28 °C and an initial pH around 5–6.

Optimization of inulinase and invertase fermentation medium by one factor at a time method and Taguchi methodology

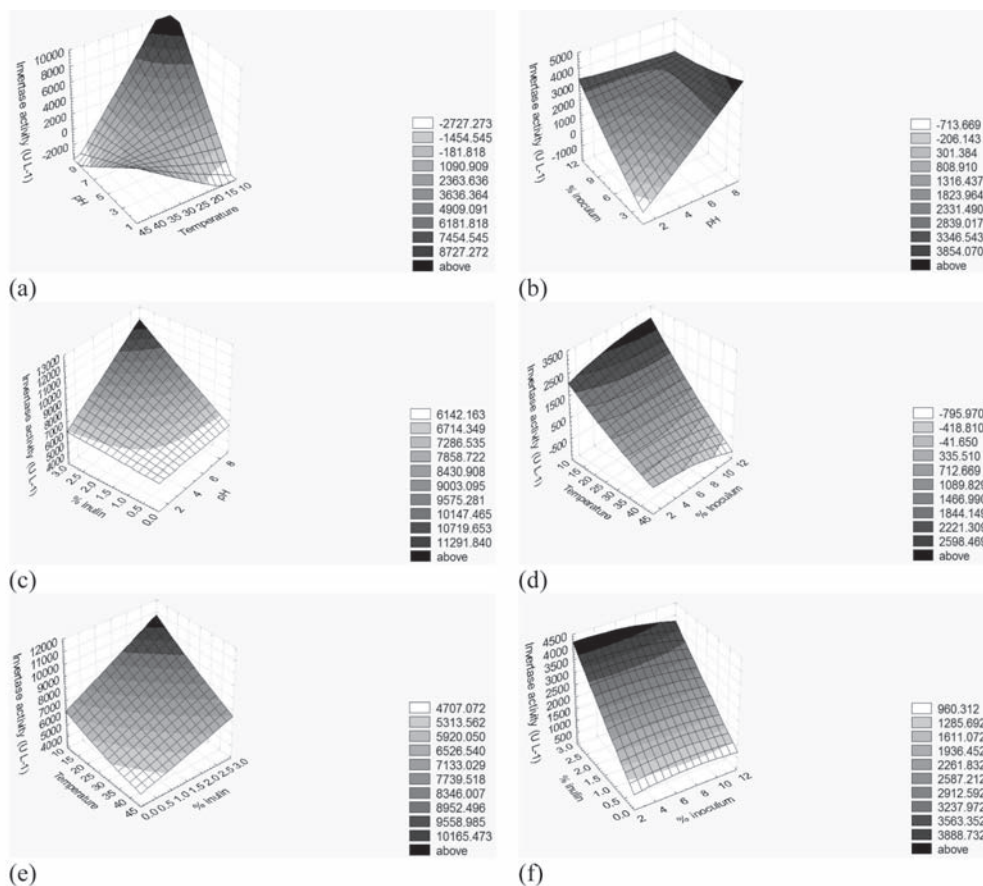


Figure 2 Response surface for the effect of factor on inulinase activity after 48 h of incubation: (a) temperature and pH at 6% inoculum and 1.5% inulin; (b) pH and inoculum at 30 °C and 1.5% inulin; (c) pH and substrate concentration at 6% inoculum and 30 °C; (d) inoculum and temperature at pH 5 and 1.5% inulin; (e) substrate concentration and temperature at 6% inoculum and pH 5; and (f) inoculum and substrate concentration at pH 5 and 30 °C.

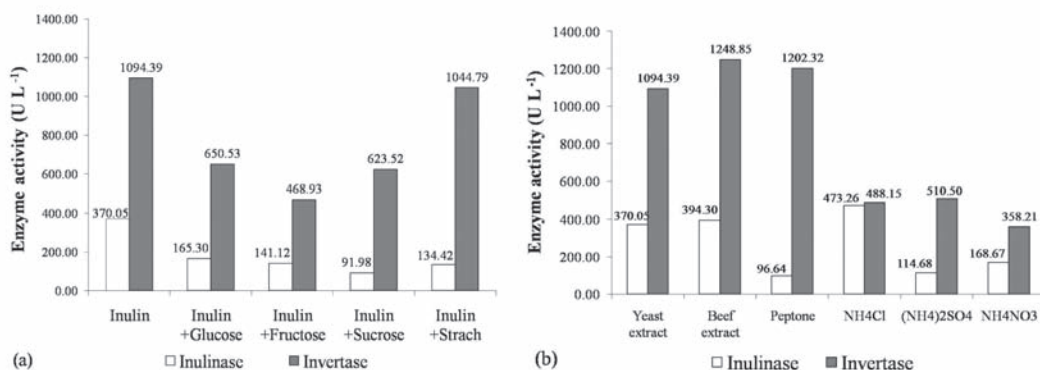


Figure 3 Effect of different (a) carbon and (b) nitrogen sources on enzymes production by *C. guilliermondii* TISTR 5844.

One factor at a time method

Glucose, fructose, sucrose and starch were used as supplements for inulin at the same concentration. Figures 3(a) and 3(b) document the effect of different carbon and nitrogen sources on enzyme production, respectively. It was observed that maximum inulinase and invertase activities of 370.05 and 1,094.39 U L⁻¹, respectively, were obtained with inulin as the sole carbon source. Hence, inulin was selected as the best carbon source. The use of inulin as the sole carbon source is known to enhance production of inulinase by *Kluyveromyces marxianus* YS-1 (Singh and Bhermi, 2008) and *Pichia guilliermondii* (Gong *et al.*, 2007). Apparently, the production of inulinase is induced or even repressed by some of the carbon sources other than inulin (Looten *et al.*, 1987; Selvakumar and Pandey, 1999; Singh and Bhermi, 2008).

An inorganic nitrogen source produced low invertase activity while the maximum invertase activity of 1,248.85 U L⁻¹ was obtained using an organic nitrogen source of beef extract. Unexpectedly, inorganic nitrogen sources like NH₄Cl produced the maximum inulinase activity of 473.26 U L⁻¹, while all the tested organic nitrogen sources resulted in high invertase activity. However, this study focused on the production of inulinase; thus, NH₄Cl was selected as the best nitrogen source.

Taguchi methodology

The experimental data obtained were processed using the Qualitek-4 version 15.1 software (QT4; Nutek Inc., MI, USA) with “bigger is better” characteristics for the determination of the optimum culture media for fermentation, to identify the influence of individual factors on the production of enzymes and to estimate the optimum conditions for fermentation as shown in Table 2. Figure 4 represents the main effect plots (indicating how each factor affects the response characteristic) for the systems. It can be seen that for each of the four variables at three levels, one level increased the mean signal-to-noise ratio compared to other levels. The optimum factor/level combination for maximizing the inulinase activity, yield ($Y_{p/s}$) and productivity (Q_p) was A₁B₂C₃D₂ (Figure 4(a)–(c)) whereas that for maximizing invertase activity was A₃B₃C₂D₃ (Figure 4(d)). Figures 4(e) and 4(f) show that the maximum invertase yield and productivity were produced by A₁B₂C₂D₃. Table 6 documents the optimized medium composition for the production of inulinase and invertase and indicates that the same conditions (inulin concentration 1%, NH₄Cl concentration 2.4%, MgSO₄·7H₂O concentration 1.2% and pH 5) achieved high inulinase activity, yield and productivity. Unexpectedly, two different conditions were observed to produce high invertase activity (5% inulin concentration, 4.8%

Table 7 Optimized media for the production of inulinase and invertase.

Variable	Optimized medium for enzyme production					
	Inulinase			Invertase		
	Activity	$Y_{p/s}$	Q_p	Activity	$Y_{p/s}$	Q_p
	(U L ⁻¹)	(U per g inulin)	(U L ⁻¹ h ⁻¹)	(U L ⁻¹)	(U per g inulin)	(U L ⁻¹ h ⁻¹)
Inulin conc. (%)	1	1	1	5	1	1
NH ₄ Cl conc. (%)	2.4	2.4	2.4	4.8	2.4	2.4
MgSO ₄ ·7H ₂ O conc. (%)	1.2	1.2	1.2	0.6	0.6	0.6
pH	5	5	5	6	6	6

conc. = concentration

$Y_{p/s}$ = yield based on inulin

Q_p = productivity of enzyme

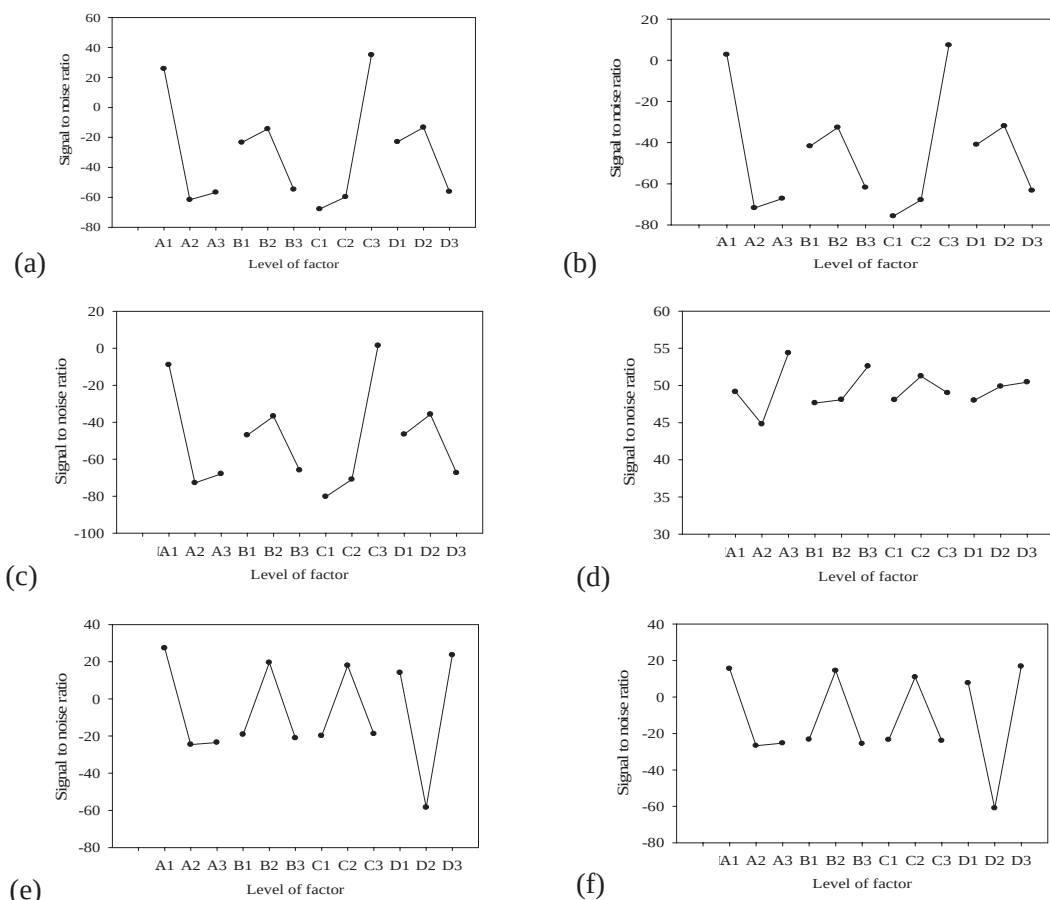


Figure 4 Main effect plots for inulinase production: (a) inulinase activity; (b) inulinase yield ($Y_{p/s}$); (c) inulinase productivity (Q_p) and for invertase production: (d) invertase activity; (e) invertase yield ($Y_{p/s}$); (f) invertase productivity (Q_p).

NH_4Cl concentration, 0.6% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ concentration and pH 6), yield and productivity (1% inulin concentration, 2.4% NH_4Cl concentration, 0.6% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ concentration and pH 6).

CONCLUSION

C. guilliermondii TISTR 5844 was found to be an efficient inulinase and invertase producer. The determination of the optimum culture conditions and media composition by RSM and Taguchi methodology led to an increase in enzyme

yield. Both experimental design approaches for process optimization involved the study of a given system by a set of independent factors over a specific region of interesting levels by identifying the influence of individual factors.

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