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Optimization of Inulinase, Invertase and β-fructofuranosidase Production from *Aspergillus niger* TISTR 3570 by the Taguchi Method

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ABSTRACT

The medium conditions for inulinase, invertase and β-fructofuranosidase (bFFase) production from *Aspergillus niger* TISTR 3570 were optimized by the Taguchi method. Production of inulinase was most affected by inulin and the production of invertase and bFFase was most affected by pH. The maximum production of enzymes was achieved by employing a medium containing: inulin (50 $g.L^{-1}$), yeast extract (12 $g.L^{-1}$), $MgSO_4 \cdot 7H_2O$ (6 $g.L^{-1}$) and an initial pH of 6. The inulinase was thermostable over a wide temperature range (25–80 ºC) and showed maximum activity at 70 ºC. In addition, activity was stable over the range pH 4–10 and the optimum for enzyme activity was pH 5. In contrast, invertase and bFFase were stable below 55 and 50 ºC, respectively, but showed highest activity at 65 and 60 ºC, respectively. Both enzymes were stable over the range pH 4–9 and had optimum activity at pH 4 for invertase and pH 5 for bFFase.

Keywords: inulinase, invertase, β-fructofuranosidase, *Aspergillus niger*, optimization, Taguchi method

INTRODUCTION

Inulin is present as a reserve carbohydrate in the roots and tubers of plant such as the Jerusalem artichoke, chicory and dahlia. It consists of linear chains of β-2,1-linked D-fructofuranose molecules terminated by a glucose (through a sucrose-type) linkage at the reducing end (Chi *et al.,* 2009). Inulin has attracted considerable research attention because it is an abundant substrate for the production of fructose-rich syrups, as well as a source for the production of fructo-oligosaccharides (FOS) and inulo-oligosaccharides (IOS), which are low caloric saccharides, acting as a growth factor for beneficial microorganisms in the intestinal flora (Skowronek and Firedurek, 2004; Yuan *et al.,* 2006). FOS can be produced from inulin by microbial enzymes having hydrolytic and transfructosylating activity. The two types of inulinase and invertase are hydrolytic enzymes. The exo-inulinase (β-D-fructohydrolase, EC 3.2.1.80) catalyzes the removal of terminal fructose residues from the non-reducing end of the inulin molecule and endo-inulinase (2, 1-β-fructan fructanohydrolase, EC 3.2.1.7) hydrolyzes the internal linkages in inulin to yield inulo-oligosaccharides (Fernandez *et al.,* 2004; Skowronek and Firedurek , 2004; Chi

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et al., 2009), and invertase breaks down sucrose to fructose and glucose by catalyzing the hydrolysis of terminal non-reducing β-fructofuranoside residues in β-fructofuranosides. While β-fructofuranosidase (bFFase) is a transfructosylating enzyme, it can transfer the fructosyl residue to the sucrose molecule at a high concentration of sucrose, in which fructosyl residues are transferred to sucrose by β-2,1 glycosidic bonds (Rubio and Navarro, 2006; Kurakake *et al.,* 2010). Most of these enzymes have been found in molds such as *Aspergillus* sp., *Fusarium* sp. and *Aureobasidium* sp.

Microbes are the best source for commercial production of enzymes because of their easy cultivation and high yield of the enzyme (Sirisansaneeyakul *et al.,* 2007a; Chi *et al.,* 2009; Songpim *et al.,* 2011). Since many enzymes of industrial significance are regulated by the composition of the medium, the study of this regulation is important in the commercial production of such enzymes. Optimization of media compositions to have a balanced proportion is very important to obtain optimum microbial growth and enzyme yield. In conventional production optimization procedures, the impact of a particular parameter is determined by altering it by set amounts while keeping the other parameters constant. Although several processes have been optimized using this methodology, such optimization procedures are time consuming and cannot provide information on the mutual interactions of the parameters on the desired outcome. Statistical procedures have advantages over conventional methodologies in predicting accurate results basically due to the utilization of the fundamental principles of statistics, randomization, replication and duplication (Rao *et al.,* 2004). One of the more interesting optimization procedures is the Taguchi method (Roy, 1990; Roy, 2001).

The Taguchi method of orthogonal array (OA) experimental design (DOE) involves the study of any given system by a set of independent variables (factors) over a specific region of interest (levels) (Mitra, 1998; Roy, 1990). Unlike traditional DOE, which focuses on the average process performance characteristics, it concentrates on the effect of variation on the process characteristics (Phadke, 1989; Ross, 1996) and makes the product or process performance insensitive to variation by the proper design of parameters. This approach also facilitates the identification of the influence of individual factors, establishing the relationship between variables and operational conditions, and finally establishing performance at the optimum levels obtained with a few well-defined experimental sets (Prasad and Mohan*,* 2005; Sirisansaneeyakul *et al.,* 2007b). Therefore, the main objective of the present work was concerned with the improvement of the inulinase, invertase and β-fructofuranosidase producing ability of *Aspergillus niger* TISTR 3570 by optimizing the medium compositions using a Taguchi methodology and partial characterization of the properties of these enzymes.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The *Aspergillus niger* TISTR 3570 used in this study was isolated from Jerusalem artichoke tubers (Sirisansaneeyakul *et al.,* 2007a) and was identified by the *Thailand Institute of Scientific and Technological Research*. The spore suspensions used as an inoculum were obtained on media agar slant containing inulin 10 $g.L^{-1}$, yeast extract 12 $g.L^{-1}$, $MgSO_4 \cdot 7H_2O$ 2 $g.L^{-1}$ and agar 15 $g.L^{-1}$ in 0.1 M McIlvain buffer pH 5 at room temperature for 1 wk. The harvesting of spores from the slants was carried out using 5 mL of sterile distilled water. The liquid medium, as described previously (50 mL) was inoculated with 5 mL of spore suspension (about 1×10^6 spores mL^{-1}) and cultured at 30 ºC on a rotary shaker with agitation at 200 rpm for 24 hr.

Optimization of medium composition by Taguchi methodology

The Taguchi methodology was used to investigate the relationship between variables of medium components and to optimize their concentrations for inulinases, invertase and β-fructofuranosidase production by *A. niger* TISTR 3570. The three levels of variation of factors that were considered and the size of experimentation were represented by symbolic arrays (L_9). Four variables with three concentration levels were used. The variables optimized were the concentrations of inulin, yeast extract and $MgSO_4 \cdot 7H_2O$, and the pH level. The experimental set is shown in Table 1. Submerged fermentation experiments were carried out in 250 mL Erlenmeyer flasks (10% v/v inoculum) containing 100 mL media as shown in Table 1 (inulin, 10, 30 and 50 $g.L^{-1}$, yeast extract, 12, 24 and 36 $g.L^{-1}$, and $MgSO_4 \cdot 7H_2O$, 2, 4 and 6 $g.L^{-1}$) in 0.1 M McIlvaine buffer (pH 4, 5 and 6) at 30 ºC on a rotary shaker with agitation at 200 rpm. After 48 hr of incubation, each flask was assayed for enzyme activity and concentration of inulin based on the total carbohydrate by the phenol sulfuric method (Dobois *et al.,* 1956).

Enzyme assay

A. niger TISTR 3570 (Sirisansaneeyakul *et al.,* 2007a) was used to simultaneously produce inulinase, invertase and β-fructofuranosidase in a batch fermentation process. The activities of inulinase and invertase were measured as previously specified (Sirisansaneeyakul *et al.,* 2007a). One unit of inulinase activity was defined as the quantity of the enzyme that liberated 1 μmol of fructose in 1 min in a 0.5% w/v solution of inulin in 0.5 M McIlvaine buffer at pH 5.0 and 40 ºC. One unit of invertase activity was defined as the quantity of the enzyme that liberated 1 μmol of fructose (or glucose) in 1 min in a 0.5% w/v (g/100 mL) solution of sucrose in 0.5 M McIlvaine buffer at pH 5.0 and 40 ºC. The activity of β-fructofuranosidase (bFFase) was measured as the reaction mixture (0.2 mL crude extract, 2.3

Table 1 Factors and their levels assigned to different columns and the orthogonal array of L_9 (3^4) design for enzyme production from *A. niger* TISTR 3570 by the Taguchi method.

Levels	A: Initial concentration of inulin ($g.L^{-1}$)	B: Initial concentration of yeast extract ($g.L^{-1}$)	C: Initial concentration of $MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	D: Initial pH
1	10	12	2	4
2	30	24	4	5
3	50	36	6	6

Experiment no.	L_9 (3^4)							
	A	B	C	D	Inulin ($g.L^{-1}$)	Yeast extract ($g.L^{-1}$)	$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	pH
1	1	1	1	1	10	12	2	4
2	1	2	2	2	10	24	4	5
3	1	3	3	3	10	36	6	6
4	2	1	2	3	30	12	4	6
5	2	2	3	1	30	24	6	4
6	2	3	1	2	30	36	2	5
7	3	1	3	2	50	12	6	5
8	3	2	1	3	50	24	2	6
9	3	3	2	1	50	36	4	4

mL 0.05 M McIlvaine buffer pH 5.0 and 7.5 mL 60% w/v (g/100 mL) solution of sucrose in 0.5 M McIlvaine buffer pH 5.0) was carried out at 55 ºC. The reducing sugar was subsequently analyzed using 3, 5-dinitrosalicylic acid (Miller, 1959). One unit of β-fructofuranosidase was defined as 1 μmol of glucose liberated in 1 min under assay conditions.

Statistical analysis

Qualitek 4 software (Nutek Inc., Bloomfield Hills, MI, USA) for the automatic design and analysis of Taguchi experiments was used to study the following objectives of the analysis. A level of $P < 0.001$ was chosen to determine significance.

Fermenter studies

After optimization studies in shake flasks, production was studied under controlled conditions in a 5 L fermenter with 3 L working volume as previously described (Sirisansaneeyakul *et al*., 2007a).

pH optimum and stability of inulinase, invertase and β-fructofuranosidase

The pH profiles of the inulinase, invertase and β-fructofuranosidase (bFFase) were evaluated by incubating the crude enzyme at pH values from 3 to 10 using the standard assay conditions in the appropriate buffer: pH 3.0–8.0 (0.5 M McIlvaine buffer) and pH 8.0–10.0 (0.5 M NH_4Cl-NH_4OH buffer). The pH stability was tested by pre-incubating the crude enzyme for 60 min at 4 ºC in 0.05 M of buffers with the same pH values from 3 to 10; the activity of the remaining enzymes was measured immediately using the standard method as described above.

Temperature optimum and stability of the inulinase, invertase and β-fructofuranosidase

The optimum temperature for the inulinase, invertase and β-fructofuranosidase (bFFase) was determined using temperatures of 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 ºC and the standard assay conditions as described above. The temperature stability of the crude enzyme was tested by pre-incubating it at a temperature from 25–80 ºC for 60 min and the residual activity was measured immediately using the standard method as described above.

RESULTS AND DISCUSSION

Optimization by Taguchi method

The experimental data (Table 2) was processed using the Qualitek-4 software with the larger-the-better attribute selected for establishing the optimum composition of the fermentation medium and identifying the individual factors that influenced enzyme production. Analysis of variance was used to analyze the results of the experiment and to determine how much variation was contributed by each factor. The percentage contributions of each factor to the production of inulinase, invertase and bFFase are shown in Tables 3–5, respectively. Statistical analysis of the inulinase production in the experimental data revealed that among all the selected factors, the initial concentration of inulin had the most impact on overall inulinase production; inulin was the most significant factor for inulinase production (Table 3). This indicated that inulin was essentially used as carbon source for inulinase production. It has been reported to be helpful for inulinase production by *Aspergillus niger* AUP19 at 50 $g.L^{-1}$ (Kumar *et al*., 2005). However, the concentration of inulin at 114.7 $g.L^{-1}$ was optimal for inulinase production by *A. ficuum* JNSP5-06 (Chen *et al*., 2011). Also, this showed that inulinase was inducible by inulin.

The percentage contributions of the factors to the production of invertase are shown in Table 4. Table 5 shows that the initial pH was the most significant factor for invertase production, bFFase activity and bFFase productivity (Q_p). This information was used to identify the optimum fermentation conditions provided by prediction

(Table 6). The relevant main effect plots are shown in Figure 1. A main effect plot reveals how the changes in a factor level affect the response of the fermentation process. For four factors (A–D), each at three levels (1–3), only one of the levels maximized the value of the main effects (Figure 1). Figure 1 suggests that the conditions for attaining a high activity, yield and productivity of any of the three enzymes are different.

The equations given in Table 6 were used to estimate the expected activity ($Y_{expected}$) of the enzymes under various conditions. The highest predicted inulinase production (1756.1 $U.L^{-1}$, 67.3 $U.g^{-1}$ inulin and 31.7 $U.L^{-1}.hr^{-1}$) was under conditions of 50 $g.L^{-1}$ inulin, 12 $g.L^{-1}$ yeast extract, 6 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, pH 6 and 30 °C. Therefore, this set of conditions was selected and used in further experiments. In the case of invertase production, two different sets of conditions were revealed in relation to invertase. One set of conditions (30 $g.L^{-1}$ inulin, 36 $g.L^{-1}$ yeast extract, 2 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, pH 6 and 30 °C) maximized both the predicted final activity and productivity of invertase. A different set of conditions (10 $g.L^{-1}$ inulin, 36 $g.L^{-1}$ yeast extract, 6 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, pH 6 and 30 °C) maximized the predicted invertase yield based on inulin. In addition, the bFFase production had two different sets of conditions. One set of conditions (10 $g.L^{-1}$ inulin, 36.g L^{-1} yeast extract, 2 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, pH 6 and 30 °C), maximized both the predicted bFFase activity and productivity. A different set of conditions (10 g. L^{-1} inulin, 36 $g.L^{-1}$ yeast extract, 6 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, pH 6 and 30 °C) maximized the predicted bFFase yield based on inulin and was similar to the conditions that maximized the invertase yield.

Under the optimal conditions identified by the Taguchi method for maximizing the inulinase production in terms of activity, yield and productivity, the predicted value of the response parameter, (the inulinase activity), $Y_{expected}$ could be calculated using Equations 1 and 2:

Table 2 Enzyme production parameters.

Experiment no.	Activity ($U.L^{-1}$)			Yield ($U.g^{-1}$)			Productivity ($U.L^{-1}.hr^{-1}$)		
	Inulinase	Invertase	bFFase	Inulinase	Invertase	bFFase	Inulinase	Invertase	bFFase
1	247.4	114.9	1283.3	14.4	7.0	110.4	2.8	1.3	21.0
2	260.3	263.9	5353.6	12.3	23.9	621.8	2.2	4.2	108.5
3	369.0	516.8	7196.5	41.9	67.7	1017.2	6.1	9.8	146.9
4	943.3	571.5	4471.5	43.3	28.7	235.1	16.6	11.0	89.7
5	959.0	272.9	1142.8	41.5	11.7	49.1	16.9	4.8	20.1
6	792.9	515.9	6187.3	31.5	23.8	298.3	13.3	10.1	126.8
7	1684.3	289.5	1134.9	52.3	8.6	36.9	30.6	5.0	21.6
8	1640.8	695.4	6926.8	56.0	25.0	266.6	30.0	13.4	142.4
9	1061.2	255.9	413.0	31.8	7.3	10.8	19.0	4.4	6.5

Table 3 Analysis of variance of factors affecting production of inulinase.

Factor	DOF	*SS*	*MS*	*F*	*S'*	Contribution (%)	Significant
Inulinase activity ($U.L^{-1}$)							
Inulin ($g.L^{-1}$)	2	6161143.1	3080571.6	3687.8	6159472.4	88.3	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	276902	138451	165.7	275231.3	3.9	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	280515.3	140257.7	167.9	278844.6	4	$P < 0.001$
pH	2	245754.3	122877.2	147.1	244083.7	3.5	$P < 0.001$
Error	18	15036.1	835.3			0.31	
Total	26	6979350.8				100	
Yield ($U.g^{-1}$)							
Inulin ($g.L^{-1}$)	2	2653.9	1327.0	157.3	2637.0	46.2	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	14.4	7.2	0.9	0.0	0.0	-
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	1223.5	611.7	72.5	1206.6	21.2	$P < 0.001$
pH	2	1660.7	830.4	98.4	1643.9	28.8	$P < 0.001$
Error	18	151.8	8.4			3.8	$P < 0.001$
Total	26	5704.3				100.0	
Productivity ($U.L^{-1}.hr^{-1}$)							
Inulin ($g.L^{-1}$)	2	2137.5	1068.8	280.6	2129.9	86.5	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	68.0	34.0	8.3	60.4	2.5	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	130.7	65.4	17.2	123.1	5.0	$P < 0.001$
pH	2	58.5	29.2	7.7	50.9	2.1	$P < 0.001$
Error	18	68.6	3.8			4.0	
Total	26	2463.3				100.0	

DOF = Degree of freedom; *MS* = Mean sum of squares; *F* = *F*-test statistic; *S'* = Pure sum of squares.

Table 4 Analysis of variance of factors affecting production of invertase.

Factor	DOF	*SS*	*MS*	*F*	*S*'	Contribution (%)	Significant
Invertase activity ($U.L^{-1}$)							
Inulin ($g.L^{-1}$)	2	116490.0	58249.5	87.1	115161.0	13.0	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	55556.9	27778.4	41.5	54218.9	6.1	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	38785.9	19392.9	29.0	37447.9	4.2	$P < 0.001$
pH	2	663802.6	331901.3	496.1	662464.6	74.7	$P < 0.001$
Error	18	12041.8	669.0			2.0	
Total	26	886686.1				100.0	
Yield ($U.g^{-1}$)							
Inulin ($g.L^{-1}$)	2	1687.4	843.7	114.9	1672.7	19.1	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	1558.6	779.3	106.1	1543.9	17.7	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	613.5	306.8	41.8	598.8	6.9	$P < 0.001$
pH	2	4751.0	2375.5	323.5	4736.3	54.2	$P < 0.001$
Error	18	132.2	7.3			2.2	
Total	26	8742.8				100.0	
Productivity ($U.L^{-1}.hr^{-1}$)							
Inulin ($g.L^{-1}$)	2	59.0	29.5	107.7	58.4	14.9	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	25.0	12.5	45.7	24.5	6.2	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	18.4	9.2	33.6	17.8	4.5	$P < 0.001$
pH	2	286.0	143.0	522.5	285.4	72.6	$P < 0.001$
Error	18	4.9	2.8			1.8	
Total	26	393.3				100.0	

DOF = Degree of freedom; *MS* = Mean sum of squares; *F* = *F*-test statistic; *S*' = Pure sum of squares.

Table 5 Analysis of variance of factors affecting production of bFFase.

Factor	DOF	*SS*	*MS*	*F*	*S*'	Contribution (%)	Significant
bFFase activity ($U.L^{-1}$)							
Inulin ($g.L^{-1}$)	2	14637376.7	7318688.3	344	14594827.9	7.9	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	30178466.5	15089233.2	709.3	30135917.7	16.2	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	14040428.7	7020214.3	330	13997879.9	7.5	$P < 0.001$
pH	2	126680044.6	63340022.3	2977.3	126637495.8	68.1	$P < 0.001$
Error	18	382938.7	5965.4			0.3	
Total	26	185919255.1				100	
Yield ($U.g^{-1}$)							
Inulin ($g.L^{-1}$)	2	1164524.0	582262.0	367.0	1161351.2	43.8	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	450059.0	225029.5	141.9	446886.2	16.9	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	91893.9	45947.0	29.0	88721.1	3.3	$P < 0.001$
H	2	917672.1	458836.0	289.2	914499.2	34.5	$P < 0.001$
Error	18	28555.1	1586.4			1.6	
Total	26	2652704.0				100.0	
Productivity ($U.L^{-1}.hr^{-1}$)							
Inulin ($g.L^{-1}$)	2	5733.4	2866.7	369.5	5717.9	7.0	$P < 0.001$
Yeast extract ($g.L^{-1}$)	2	13725.9	6863.0	884.6	13710.4	16.8	$P < 0.001$
$MgSO_4 \cdot 7H_2O$ ($g.L^{-1}$)	2	5975.0	2987.5	385.1	5959.4	7.3	$P < 0.001$
pH	2	56204.7	28102.3	3622.4	56189.2	68.7	$P < 0.001$
Error	18	139.6	7.8			0.3	
Total	26	81778.6				100.0	

DOF = Degree of freedom; *MS* = Mean sum of squares; *F* = *F*-test statistic; *S*' = Pure sum of squares.

Table 6 Optimal enzyme conditions and comparison between predicted and experimental values at the optimal condition for inulinase production.

Parameter	Factors level [a]				Optimal conditions for production of inulinase		Predicted based on optimal conditions ($Y_{expected}$)
	A	B	C	D	Predicted	Experimental	
Inulinase							
Activity ($U.L^{-1}$)	50	12	6	6	1756.1	1438.9	$Y_{\text{expected}} = A3 + B1 + C3 + D3 - 3\overline{T}$
Yield ($U.g^{-1}$ inulin)	50	12	6	6	67.3	86.7	$Y_{\text{expected}} = A3 + B1 + C3 + D3 - 3\overline{T}$
Productivity ($U.L^{-1}.hr^{-1}$)	50	12	6	6	31.7	29.4	$Y_{\text{expected}} = A3 + B1 + C3 + D3 - 3\overline{T}$
Invertase							
Activity ($U.L^{-1}$)	30	36	2	6	527.7	600	$Y_{\text{expected}} = A2 + B3 + C1 + D3 - 3\overline{T}$
Yield ($U.g^{-1}$ inulin)	10	36	6	6	30.3	29.6	$Y_{\text{expected}} = A1 + B3 + C3 + D3 - 3\overline{T}$
Productivity ($U.L^{-1}.hr^{-1}$)	30	36	2	6	10.0	10.0	$Y_{\text{expected}} = A2 + B3 + C1 + D3 - 3\overline{T}$
bFFase							
Activity ($U.L^{-1}$)	10	36	2	6	3107.9	7047.4	$Y_{\text{expected}} = A1 + B3 + C1 + D3 - 3\overline{T}$
Yield ($U.g^{-1}$ inulin)	10	36	6	6	224.6	390.2	$Y_{\text{expected}} = A1 + B3 + C3 + D3 - 3\overline{T}$
Productivity ($U.L^{-1}.hr^{-1}$)	10	36	2	6	62.3	132.3	$Y_{\text{expected}} = A1 + B3 + C1 + D3 - 3\overline{T}$

[a] = See Table 1 for an explanation of the factors A-D

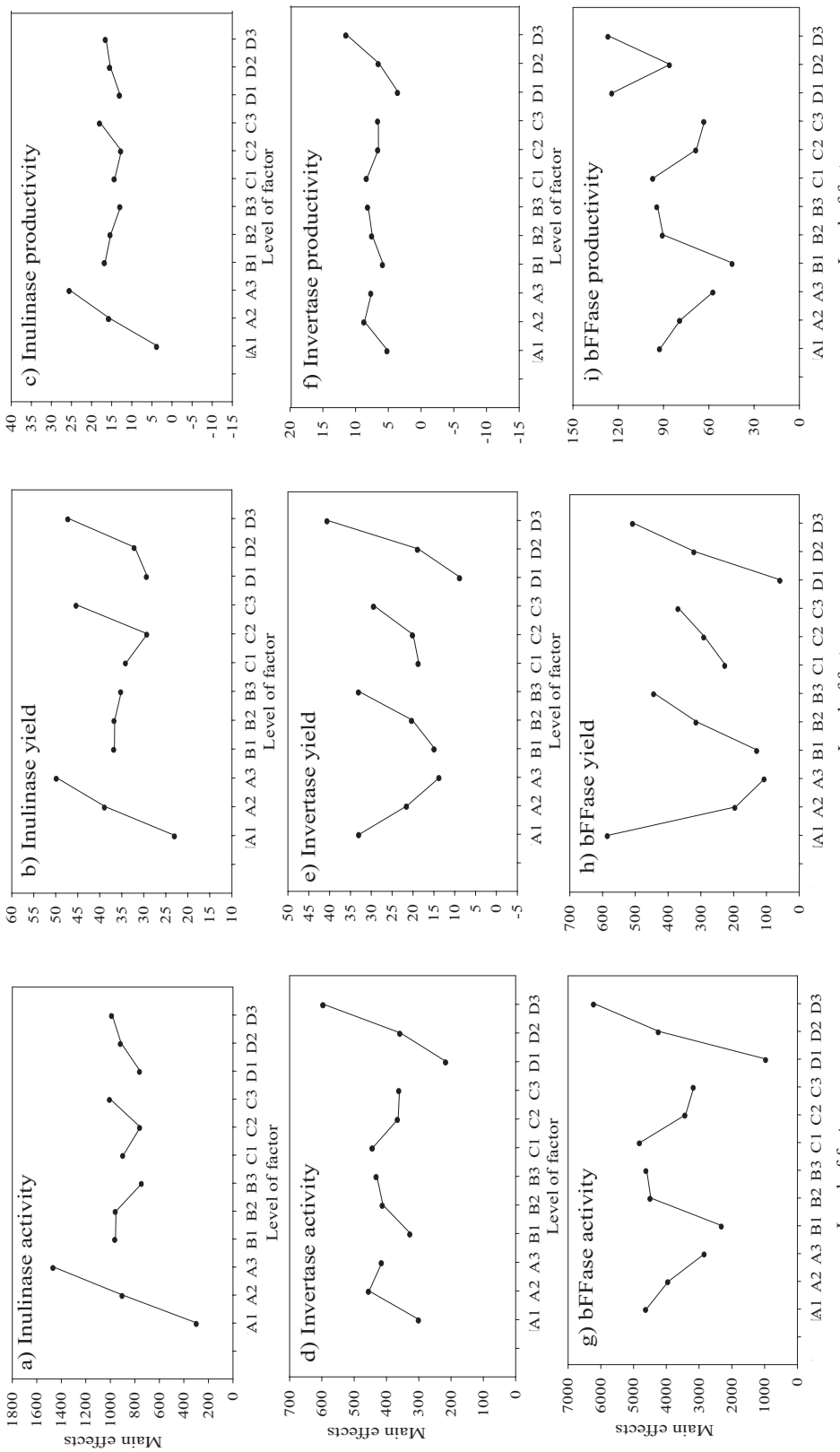

Figure 1 Main effects for various factors (A–D, Table 1) and levels (1–3, Table 1): (a) inulinase activity; (b) inulinase yield; (c) inulinase productivity; (d) invertase activity; (e) invertase yield; (f) invertase productivity; (g) bFFase activity; (h) bFFase yield; (i) bFFase productivity. Optimal conditions are indicated by the peak values of the main effects.

$$Y_{\text{expected}} = A3 + B1 + C3 + D3 - 3\bar{T} \quad (1)$$

where $\bar{T}$ is calculated using Equation 2:

$$T = \left(\sum (Average\ of\ \exp eriment)_{inulinase}\right) / N = 884.2 / 9 = 2652.8 \quad (2)$$

Equation (1) and (2) produce Equation 3:

$$Y_{\text{expected}} = (1462.1 + 958.3 + 1004.1 + 984.3) - 2652.8 = 1756.1 \quad (3)$$

Therefore, the inulinase activity, Y_{expected} is 1756.1 $U.L^{-1}$. The predicted and the expected values of Y_{expected} for the three enzymes are shown in Table 6.

Confirmation under optimal conditions

Confirmation testing is a necessary requirement of the Taguchi method (Roy, 1990; Roy, 2001). A single confirmation test was conducted for enzyme production using the above identified optimum settings of the process parameters.

The confirmation test results for the set of optimization condition (Table 6) revealed a comparable predicted value with confirmation of the enzyme activity, yield and productivity for enzyme production. The confirmation data showed the highest inulinase activity (1438.9 $U.L^{-1}$), yield based on inulin (86.7 $U.g^{-1}$ inulin) and productivity (29.4 $U.L^{-1}.hr^{-1}$) within 48 hr. This set of inulinase conditions favored the preferential production of invertase and bFFase and therefore maximized the production of both invertase and bFFase (Figure 2).

Under optimal conditions for inulinase production, the maximal production of the three enzymes occurred during growth within 72 hr (Figure 2). Inulinase activity increased continuously with the approach of the stationary phase of growth at 1907.2 $U.L^{-1}$ (Figure 2(a)). Similarly, under this condition, the activity levels of invertase (815.3 $U.L^{-1}$) and bFFase (9200.0 $U.L^{-1}$) were relative high at 72 hr (Figures 2(a) and 2(b)). The high level of inulinase attained within 72 hr under optimal conditions (Figure 2) compared well with the results for other producer microorganisms where the inulinase activity generally was reported to peak much earlier in the fermentation process. For example, for *Aspergillus niger*-245, Cruz *et al.* (1998) reported peak production (2,000 $U.L^{-1}$) after 48–60 hr of fermentation. Similarly, using *Aspergillus niger* AUP 19, Kumar *et al.* (2005) observed the highest inulinase activity after 72 hr of incubation and found that the maximum productivity of inulinase from *A. niger* AUP 19 was achieved by employing a medium containing 5% (w/v) inulin, an incubation temperature of 28 ºC and pH 6.5, with the inoculum level at 10% (v/v). Under optimal conditions of inulinase production, bFFase produced by *A. niger* TISTR 3579 gave better activity (9,200.0 $U.L^{-1}$) and productivity (118.1 $U.L^{-1}.hr^{-1}$) compared to those obtained from *A. niger* NRRL 330. On the other hand, bFFase activity and productivity resulting from *A. niger* NRRL 330 were reported as 6,996.0 $U.L^{-1}$ and 58.3 $U.L^{-1}.hr^{-1}$, respectively, which were attained at 120 hr culture time (Balasubramaniem *et al.,* 2001). Fernandez *et al.* (2004) reported 1800.0 $U.g^{-1}$ and 25.0 $U.g^{-1}.hr^{-1}$ at 72 hr culture time. This revealed that bFFase production from *A. niger* TISTR 3579 was the highest among those previously reported (Table 7).

After the enzyme production in the flask was optimized, the obtained conditions were applied for enzyme production in a 5 L fermenter. The batch was run with the same optimal conditions and an aeration rate of 1 vvm at an agitation rate of 600 rpm. Figure 3(a) shows the inulinase production in the fermenter under optimal conditions. It was possible to verify that the biosynthesis of the inulinase started after 21 hr of incubation and that the largest enzyme liberation occurred after 36 hr of fermentation. Both invertase and bFFase production occurred simultaneously with the growth of fungi, starting after 3 hr of incubation and the highest amount of enzyme was produced at 39 hr (Figures 3(a) and 3(b)).

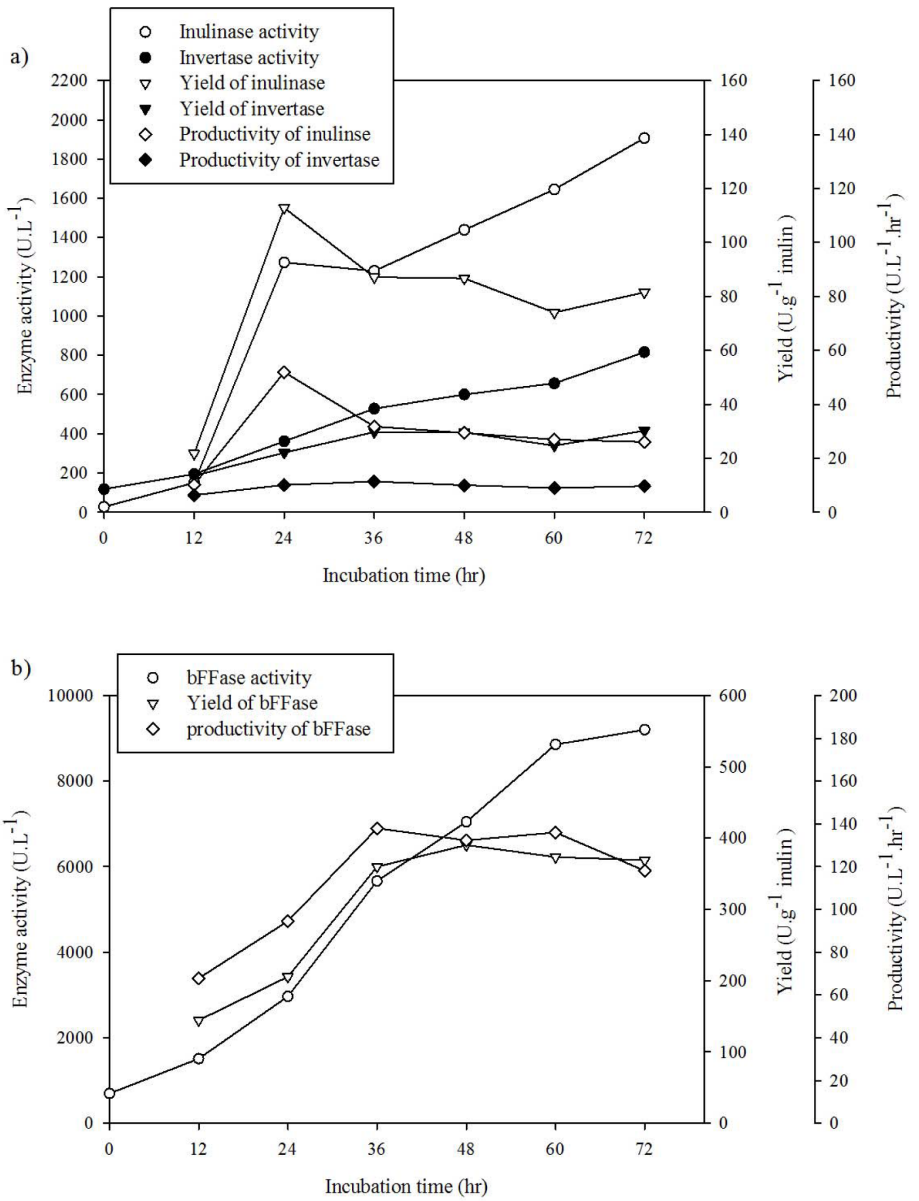

Figure 2 Yield from inulin, activity and productivity of inulinase, invertase and bFFase: (a) inulinase and invertase production; (b) bFFase production under optimal conditions (inulinase production) in 500 mL Erlenmeyer flask.

Effects of pH on enzyme activity and stability

The effects of pH on the activity and stability of enzyme activity were determined at various level of pH (3–10) under standard conditions. As shown in Table 7, inulinase and bFFase showed maximum activity at pH 5, but invertase exhibited the highest activity at pH 4.0 (Figure 4), which was in agreement with the general range of many microbial sources reported, for example, by Chen *et al.* (2009) who found the pH optimum was pH 4–5 for *Aspergillus ficuum* JNSP5-06 and by Derycke and Vandamme

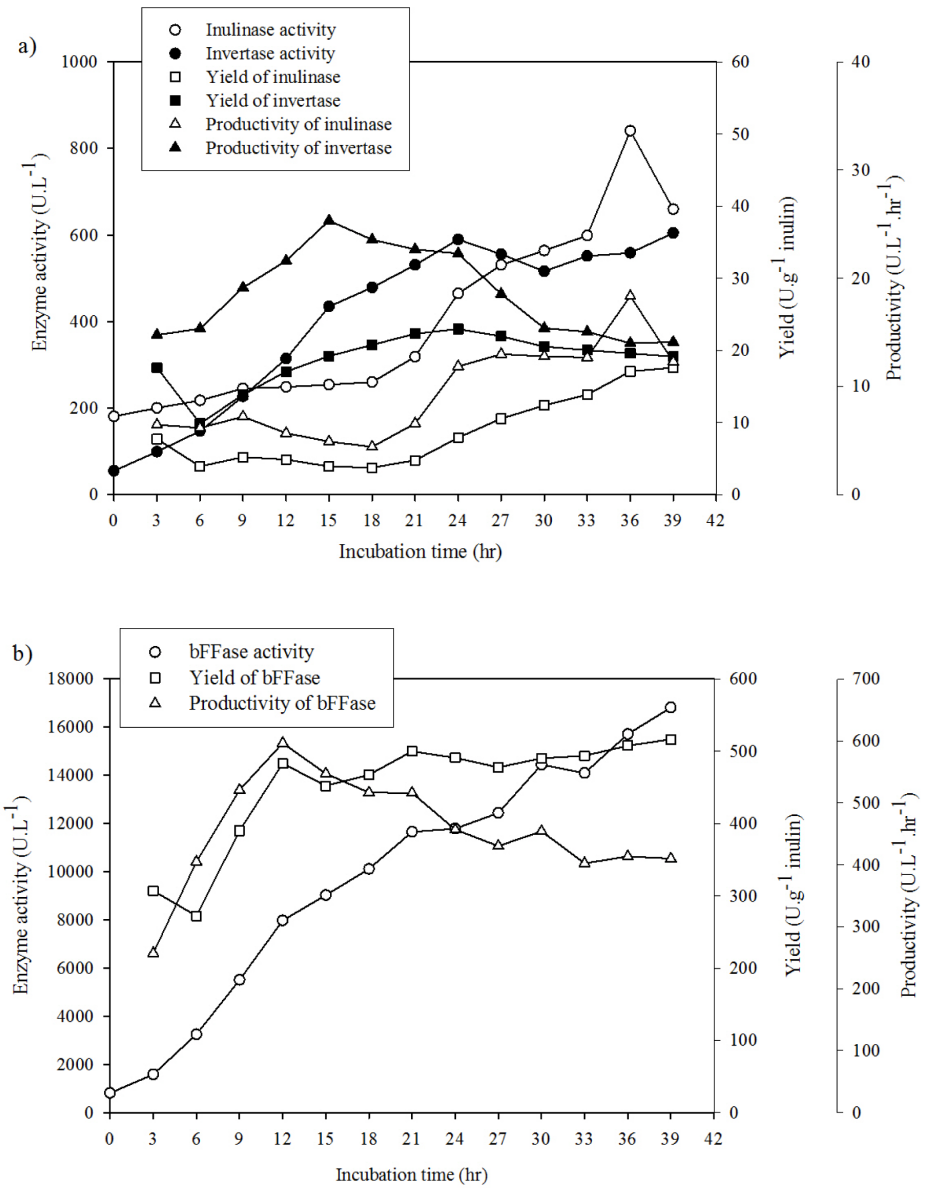

Figure 3 Yield from inulin, activity and productivity of inulinase, invertase and bFFase: (a) inulinase and invertase production; (b) bFFase production under optimal conditions (inulinase production) in a 5 L fermenter.

(1984) who reported an optimum at pH 4.4 with *Aspergillus niger*.

Under standard temperature conditions for each enzyme (40 ºC for inulinase and invertase, and 55 ºC for bFFase), the enzymes were stable over a wide pH range (from 3 to 10) at 60 min, especially, bFFase. However, the inulinase activity decreased dramatically beyond that range, and was almost inactive below pH 4.0 (Figure 4(a)). As shown in Figure 4(c), invertase activity declined below pH 4 and above pH 9.

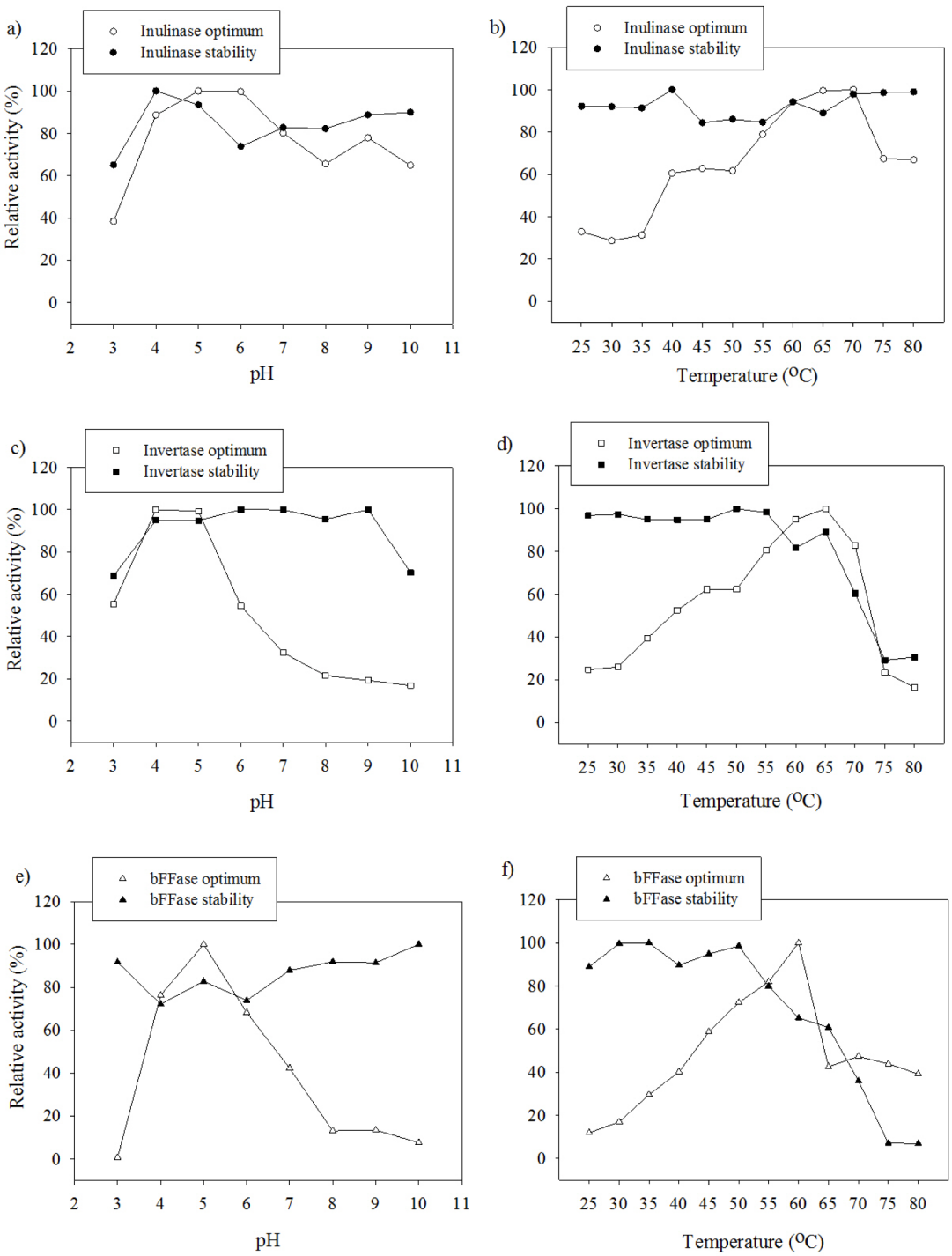

Figure 4 Effect of pH and temperature on enzyme activity and stability: (a) pH optimum and stability of inulinase; (b) temperature optimum and stability of inulinase; (c) pH optimum and stability of invertase; (d) temperature optimum and stability of invertase; (e) pH optimum and stability of bFFase; (f) temperature optimum and stability of bFFase.

Table 7 Comparison of some potential inulinase/invertase/bFFase producers.

Fungal strain	Enzyme	Activity ($U.L^{-1}$)	Productivity ($U.L^{-1}.hr^{-1}$)	Optimum	Stability	Reference
Aspergillus niger TISTR 3570	Inulinase	120.2	0.75	40 °C (pH 5)	-	Sirisansaneeyakul *et al*. (2007a)
	Invertase	634.8	5.29	40 °C (pH 5)	-	
A. niger TISTR 3570	Inulinase	1907.2	31.7	70 °C (pH 5)	25–80 °C (pH 4–10)	This study
	Invertase	815.3	9.7	65 °C (pH 4)	25–55 °C (pH 4–9)	This study
	bFFase	9200.0	118.1	60 °C (pH 5)	25–50 °C (pH 3–10)	This study
A. ficuum JNSP5-06	Inulinase	$25x10^3$	210	45 °C (pH 4–5)	<50 °C (pH 4–8)	Jing *et al.*(2003); Chen *et al.* (2009)
	Invertase	$20x10^3$	170	-	-	
A. niger AUP 19	Inulinase	$176 x10^3$	2440	-	-	Kumar *et al.* (2005)
Penicillium sp. TN-88	Inulinase	$9.9x10^3$	110	50 °C (pH 5.2)	-	Nakamura *et al.* (1997)
A. niger NRRL 330	bFFase	6996.0	58.3	-	-	Balasubramaniem *et al.* (2001)
A. ochraceus	bFFase	$108x10^3$	$1.12x10^3$	60 °C (pH 4.5)	-	Guimarees *et al.* (2007)
Aspergillus sp. 27H	bFFase	1800.0[a]	25.0[b]	60 °C (pH 5.5–6)	30–60 °C (pH 5.5–8)	Fernandez *et al.* (2004)

[a] = $U.g^{-1}$; [b] = $U.g^{-1}.hr^{-1}$.

Effect of temperature on enzyme activity and stability

To determine the optimum temperature for enzyme activity, enzyme reactions were performed at various temperatures (25–80 ºC) at pH 5.0 under standard conditions. As shown in Table 5, the optimum temperatures of inulinase, invertase and bFFase were obtained at 70, 65 and 60 ºC, respectively. To determine the stability of each enzyme, an enzyme solution was incubated at various temperatures (25–80 ºC) at pH 5.0 for 60 min. After the treatment, the residual activities were measured at standard temperatures (under standard conditions). Inulinase retained around 80% relative activity after 60 min (Figure 4(b)) and invertase retained around 90% relative activity after 60 min but was almost inactive above 55 ºC (Figure 4(d)). As shown by Figure 4(f), bFFase was stable over a wide temperature range (from 25 to 50 ºC) after 60 min. It was found that the stability of bFFase activity was lower above 50 ºC. For inulin hydrolysis, thermal enzyme stability was used as a key factor for industrial application. For example, a reaction temperature of 60 ºC is usually used to increase productivity and to avoid possible contamination. A few different inulinases from different sources could comply with such goal (Panesar *et al.*, 2010). Therefore, inulinase from *A. niger* TISTR 3570 would be regarded as a thermostable enzyme and could be successfully used to hydrolyze inulin at a relatively high temperature.

CONCLUSION

Aspergillus niger TISTR 3570 was found to be a good producer of inulinase, invertase and β-fructofuranosidase. The yields of the enzymes were enhanced substantially by optimization of the medium composition. Conditions were established for preferential production of the three enzymes. Under optimal conditions, the titers of the enzymes in the present study were generally consistent with the results attained in fermentation studies with other microorganisms (Table 7). With its ability to produce enzymes in a minimally controlled batch fermentation process and being characterized as thermostable for these enzymes, *A. niger* TISTR 3570 is potentially useful for producing a commercial enzyme cocktail readily utilizable for making either sweeteners or prebiotics from inulin.

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