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# Extraction optimization of inulinase obtained by solid state fermentation of *Aspergillus ficuum* JNSP5-06

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ABSTRACT

Production of inulinase by solid state fermentation always involves an extraction step. In this study, the effects of the type of solvent, solid/liquid ratio, stirring rate, temperature, and extraction time on the recovery of inulinase from solid state fermentation of *Aspergillus ficuum* JNSP5-06 were investigated. After determining the best solvent, the extraction parameters for inulinase recovery were optimized by central composite rotatable design. The results showed that the maximum recovery of inulinase was obtained using 0.1 M sodium acetate of pH 4.5 as extraction solvent, in a solid/liquid ratio of 1:20.5, at 39.6 °C and 207 rpm for 40 min.

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## 1. Introduction

Inulinases are potentially useful enzymes for the production of fructooligosaccharides and high fructose syrups from inulin (Ettalibi & Baratti, 1987). Fructooligosaccharides have good functional and nutritional properties, such as low calorie diet, *Bifidobacteria* stimulating factor, and source of dietary fibre in food preparations. Fructose production by inulin hydrolysis is more advantageous than conventional process based on starch, which includes the action of α-amylase, amyloglucosidase and glucose isomerase, yielding only 45% of fructose (w/w) in the final product due to the thermodynamical equilibrium of the reaction. However, hydrolysis of inulin by inulinase can yield products with 95% of fructose (w/w) (Kim & Rhee, 1989).

Traditionally, inulinase is produced by submerged fermentation (Gill, Sharma, Harchand, & Singh, 2003; Kalil, Suzan, Maugeri, & Rodrigues, 2001; Selvakumar & Pandey, 1999a). However, in recent years, inulinase production by solid state fermentation (SSF) has attracted more attention (Bender, Mazutti, Oliveira, Di Luccio, & Treichel, 2006; Chen, Wang, & Li, 2007; Mazutti, Bender, Treichel, & Di Luccio, 2006; Selvakumar & Pandey, 1999b).

SSF processes present a series of advantages over submerged fermentations. The culture conditions are more similar to the natural habitat of filamentous fungi, so that these are able to grow and excrete large quantities of enzymes. Product concentrations after extraction are usually larger than those of products obtained by submerged fermentation and the quantity of liquid waste generated is lower. Additionally, these processes are of special economic interest for countries with abundance of biomass and agroindustrial residues, as these can be used as cheap raw materials (Castilho, Medronho, & Alves, 2000).

However, one of the important aspects of SSF is the adequate recovery of enzymes from the fermented solids (Castilho, Alves, & Medronho, 1999). According to Ikasari and Mitchell (1996), extraction efficiency is a critical factor determining the economic feasibility of SSF for enzyme production. In order to make SSF applicable for the production of high-purity enzymes, some studies are found about extraction of enzymes in SSF. Castilho et al. (1999, 2000) investigated extraction of pectinases obtained by SSF, verifying the influence of temperature, stirring rate, and time of extraction in batch extraction and in fixed-bed, multiple-stage extraction. Díaz, Caro, Ory, and Blandino (2007), Heck, Hertz, and Ayub (2005) and Pal and Khanum (2010) studied extraction of xylanases obtained by SSF using central composite design, indicating the combined influence of solvent nature, temperature, time of contact, agitation and solid/liquid ratio. Bender, Mazutti, Di Luccio, and Treichel (2008) also investigated the effects of the kind of solvent, temperature, stirring rate, pH, and solid/liquid ratio on inulinase extraction from fermented sugarcane bagasse. However, so far, very limited investigations about the extraction of inulinase obtained by SSF have been reported.

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**Table 1**
Effects of different solvents on inulinase recovery.

| Solvent | Inulinase activity (U/gds) |
|---|---|
| 0.1 M Sodium citrate buffer pH 3.0 | 122.7 |
| 0.1 M Sodium citrate buffer pH 3.5 | 131.4 |
| 0.1 M Sodium acetate buffer pH 4.0 | 121.6 |
| 0.1 M Sodium acetate buffer pH 4.5 | 150.9 |
| 0.1 M Sodium acetate buffer pH 5.0 | 143.8 |
| 0.1 M Sodium acetate buffer pH 5.5 | 140.5 |
| 0.1 M Sodium phosphate buffer pH 6.0 | 130.2 |
| 0.1 M Sodium phosphate buffer pH 7.0 | 122.2 |
| 0.1 M Sodium phosphate buffer pH 8.0 | 114.8 |
| NaCl 10% (w/v) | 133.8 |
| NaCl 20% (w/v) | 122.7 |
| Ethanol 10% (v/v) | 130.8 |
| Ethanol 20% (v/v) | 109.5 |
| Distilled water | 116.3 |

Results are the mean of three experiments.

The purpose of this study was to investigate the extraction conditions of inulinase obtained by SSF of wheat bran and *Aspergillus ficuum* JNSP5-06. The effects of the type of solvent, solid/liquid ratio, stirring rate, temperature, and extraction time on recovery of inulinase were studied using an experimental design technique.

## 2. Materials and methods

### 2.1. Microorganism and medium

*A. ficuum* JNSP5-06 was isolated from a soil sample (Chen et al., 2009b). The strain was maintained on agar slant medium at 4 °C. The medium contained the following components (g/L): tomato 200, glucose 15, agar 20.

### 2.2. Inoculum preparation

Inocula were prepared in a medium containing (g/L) yeast extract 10, peptone 20 and inulin 20. A loop of cells from the slant were transferred into a 250 mL conical flask containing 30 mL culture medium and incubated for 24 h on a rotary shaker operating at 200 rpm at 30 °C.

### 2.3. Solid-state fermentation

Wheat bran purchased from the local market was used as the solid substrate for inulinase production. Ten g of wheat bran was supplemented with 13 mL of distilled water containing 11.47% inulin, 0.76% $NH_4H_2PO_4$, 0.5% NaCl, 0.05% $MgSO_4$, 0.01% $ZnSO_4$, 0.1% $KH_2PO_4$, 5.71% corn steep liquor, 0.5% Tween-80 on the basis of dry solid substrate (w/w). The substrates were set at initial pH 5.5 in 250 mL conical flasks, sealed with hydrophobic cotton and autoclaved at 121 °C for 30 min. The cooled substrates were inoculated with a 4% inoculum level, mixed carefully under strictly aseptic conditions with sterile glass rods, and then incubated in a chamber with relative humidity above 80% at 28 °C for 72 h in a static mode.

### 2.4. Extraction of inulinase

When fermentation was completed, a weighed quantity of the fermented matter was transferred to 250 mL conical flasks with the addition of 20 volumes of extraction solvent (w/v, based on initial dry weight of the substrate) and the mixture was mixed thoroughly on a rotary shaker (150 rpm) at room temperature (20 ± 2 °C) for 60 min. The mixtures were filtered through muslin cloth. After centrifugation of the filtrate at 1503 × *g* and at 4 °C for 10 min, the supernatant was collected as the crude enzyme solution and the total volume was recorded.

### 2.5. Inulinase activity assay

The inulinase activity was determined by measuring the reducing sugars released from the hydrolysis of sucrose. Briefly, 0.2 mL of suitably diluted enzyme extract was mixed with 2 mL of sucrose solution (2%, w/w) in 0.2 mol/L acetate buffer (pH 4.5). The reaction was carried out at 50 °C for 30 min and then terminated by boiling for 5 min. The reducing sugar concentration of the reaction mixture was analyzed by DNS method (Kalil et al., 2001). One unit of inulinase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugar per minute under the assay conditions used in this study. The results are expressed in terms of units per gram of dry substrate (U/gds).

### 2.6. Selection of extraction solvent for inulinase

Different solvents were tested for extraction of inulinase: distilled water, 0.1 M sodium citrate buffer (pH 3.0, 3.5), 0.1 M sodium acetate buffer (pH 4.0, 4.5, 5.0, 5.5), 0.1 M sodium phosphate buffer (pH 6.0, 7.0, 8.0), NaCl solution (10% and 20%, w/v), and aqueous ethanol solution (10% and 20%, v/v). The kinds of solvents, the range of pH and concentration were chosen based on some previous studies of extraction of inulinase (Bender et al., 2008), pectinase (Castilho et al., 2000), xylase (Díaz et al., 2007; Heck et al., 2005; Pal & Khanum, 2010), protease (Aikat & Bhattacharyya, 2000; Tunga, Banerjee, & Bhattacharya, 1999), and our previous preliminary experiments with the inulinase.

2 g fermented matter was transferred to 250 mL conical flasks with the addition of 20 volumes of different solvent (w/v) and the mixture was mixed thoroughly on a rotary shaker (150 rpm) at room temperature (20 ± 2 °C) for 60 min for inulinase extraction.

### 2.7. Optimization of extraction parameters for the recovery of inulinase

A central composite rotatable design (CCRD) comprising of three variables (solid/liquid ratio (mL/gds), stirring rate (rpm), and temperature (°C)) at five levels (preliminary experiments were carried out to determine the parameters range) was used to study their interactive influence on inulinase recovery from optimized solid-state medium. The results of CCRD with six replicates at the center point were used to fit the second order response surface.

Once the experiments were performed, the experimental results were fitted with a second order polynomial function:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where $Y$ was inulinase activity, $\beta_0$ was the intercept term, $\beta_1$, $\beta_2$ and $\beta_3$ were linear coefficients, $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ were interaction coefficients, $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$ were squared coefficients, and $X_1$, $X_2$ and $X_3$ were coded independent variables.

### 2.8. Statistical analysis

Design-Expert software 7.1.4 (Stat-Ease, Inc., Minneapolis, USA) was used for the experiment design and regression analysis. The statistical significance of regression coefficients was 95%. The optimum levels of variables were obtained by graphical analysis.

## 3. Results and discussion

### 3.1. Selection of extraction solvent for inulinase

Recovery of enzyme from the solid medium was one important aspect of SSF. An ideal solvent would extract the enzyme selec-

**Table 2**
Variables and levels of central composite rotatable design.

| Independent variables | Symbol | Range and level | | | | |
|---|---|---|---|---|---|---|
| | | −1.682 | −1 | 0 | +1 | +1.682 |
| Solid/liquid ratio | $X_1$ | 11.6 | 15 | 20 | 25 | 28.4 |
| Stirring rate (rpm) | $X_2$ | 116 | 150 | 200 | 250 | 284 |
| Temperature (°C) | $X_3$ | 31.6 | 35 | 40 | 45 | 48.4 |

**Table 3**
Central composite rotatable design and results.

| Run | Variables | | | | | | Inulinase activity (U/gds) |
|---|---|---|---|---|---|---|---|
| | Coded levels | | | Actual levels | | | |
| | $X_1$ | $X_2$ | $X_3$ | $X_1$ | $X_2$ | $X_3$ | |
| 1 | −1 | −1 | −1 | 15 | 150 | 35 | 135.3 |
| 2 | −1 | −1 | 1 | 15 | 150 | 45 | 132.1 |
| 3 | −1 | 1 | −1 | 15 | 250 | 35 | 143.5 |
| 4 | −1 | 1 | 1 | 15 | 250 | 45 | 139.2 |
| 5 | 1 | −1 | −1 | 25 | 150 | 35 | 135.1 |
| 6 | 1 | −1 | 1 | 25 | 150 | 45 | 140.7 |
| 7 | 1 | 1 | −1 | 25 | 250 | 35 | 149.8 |
| 8 | 1 | 1 | 1 | 25 | 250 | 45 | 143.4 |
| 9 | −1.682 | 0 | 0 | 11.6 | 200 | 40 | 136.3 |
| 10 | 1.682 | 0 | 0 | 28.4 | 200 | 40 | 149.8 |
| 11 | 0 | −1.682 | 0 | 20 | 116 | 40 | 132.1 |
| 12 | 0 | 1.682 | 0 | 20 | 284 | 40 | 148.9 |
| 13 | 0 | 0 | −1.682 | 20 | 200 | 31.6 | 155.7 |
| 14 | 0 | 0 | 1.682 | 20 | 200 | 48.4 | 142.6 |
| 15 | 0 | 0 | 0 | 20 | 200 | 40 | 186.1 |
| 16 | 0 | 0 | 0 | 20 | 200 | 40 | 189.4 |
| 17 | 0 | 0 | 0 | 20 | 200 | 40 | 188.9 |
| 18 | 0 | 0 | 0 | 20 | 200 | 40 | 193.6 |
| 19 | 0 | 0 | 0 | 20 | 200 | 40 | 190.2 |
| 20 | 0 | 0 | 0 | 20 | 200 | 40 | 185.6 |

*Note*: $X_1$, solid/liquid ratio; $X_2$, stirring rate (rpm); $X_3$, temperature (°C). Extraction time is 60 min. Results are the mean of three experiments.

tively and completely at room temperature with minimal contact time and, preferably, at the pH of the cultivated substrate (Singh, Ramakrishna, & Rao, 1999). As shown in Table 1, among the solvents tested, 0.1 M sodium acetate buffer of pH 4.5 gave the best recovery of inulinase from the fermented solid, which was agreement with the previous study. Bender et al. (2008) reported that the highest inulinase recovery was obtained using 0.1 M sodium acetate buffer of pH 4.8 as extraction solvent. From Table 1, we also found that when extraction solvent with very high or very low pH was used, a decrease in recovery of inulinase was observed because pH directly affected enzyme stability due to inactivation. In this study, ethanol solution presents low recovery of inulinase because ethanol solution is usually good precipitating agent for protein solutions above some concentrations. Moreover, ethanol solution at high concentration can also inhibit enzyme activity by denaturation, and this may also be the cause of low value of enzyme recovery. Therefore, 0.1 M sodium acetate buffer of pH 4.5 was selected as extraction solvent of inulinase in subsequent experiments.

### 3.2. Optimization of extraction parameters for the recovery of inulinase

After selecting 0.1 M sodium acetate buffer of pH 4.5 as the best extraction solvent for inulinase, a full factorial CCRD was applied to

**Table 4**
Analysis of variance for the experimental results of the central composite rotatable design.

| Factor[a] | Standard error | Sum of square | Degree of freedom | *F*-value | *P*-value | Significance |
|---|---|---|---|---|---|---|
| $X_1$ | 0.88 | 126.74 | 1 | 12.08 | 0.0060 | ** |
| $X_2$ | 0.88 | 272. 05 | 1 | 25.93 | 0.0005 | ** |
| $X_3$ | 0.88 | 67.37 | 1 | 6.42 | 0.0297 | * |
| $X_1^2$ | 0.85 | 3960.87 | 1 | 377.57 | <0.0001 | ** |
| $X_2^2$ | 0.85 | 4403.38 | 1 | 419.75 | <0.0001 | ** |
| $X_3^2$ | 0.85 | 2997.37 | 1 | 285.72 | <0.0001 | ** |
| $X_1X_2$ | 1.15 | 0.55 | 1 | 0.053 | 0.8233 | |
| $X_1X_3$ | 1.15 | 5.61 | 1 | 0.53 | 0.4813 | |
| $X_2X_3$ | 1.15 | 21.45 | 1 | 2.04 | 0.1832 | |
| Model | | 9992.58 | 9 | 105.84 | <0.0001 | ** |
| Error | | 104.91 | 10 | | | |
| Total SS[b] | | 10097.49 | 19 | | | |

[a] $X_1$, solid/liquid ratio; $X_2$, stirring rate; $X_3$, temperature.
[b] Sum of square.
* Statistically significant at 95% of probability level.
** Statistically significant at 99% of probability level.

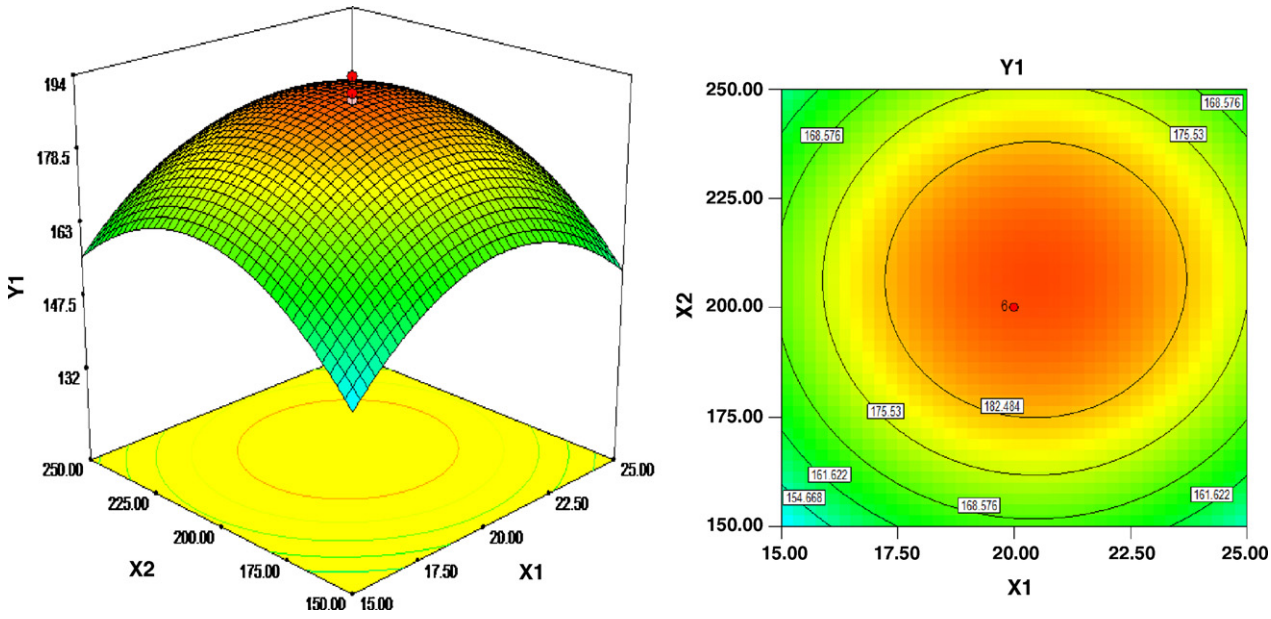


**Fig. 1.** Effects of solid/liquid ratio ($X_1$) and stirring rate ($X_2$) on the recovery of inulinase ($Y_1$).

study the interactive effects of solid/liquid ratio, stirring rate, and temperature on the recovery of inulinase and to derive a statistical model for their effects. The range of the independent variables is presented in Table 2.

A second order polynomial model was fitted to the obtained inulinase recovery. The statistical combinations of the critical components with the observed recovery of inulinase are listed in Table 3. The application of the RSM yielded the following regression equation which is an empirical relationship between the inulinase recovery and the test variables in coded units:

$$Y_1 = 189.02 + 3.05X_1 + 4.46X_2 - 2.22X_3 - 16.58X_1^2 + 0.26X_1X_2 + 0.84X_1X_3 - 17.48X_2^2 - 1.64X_2X_3 - 14.42X_3^2 \quad (2)$$

where $Y_1$ is the inulinase activity (U/gds), $X_1$ is solid/liquid ratio (gds/mL), $X_2$ is stirring rate (rpm) and $X_3$ is temperature (°C). The analysis of variance of the quadratic regression model demonstrated that Eq. (2) was a highly significant model, as was evident from the Fisher's $F$-test with a very low probability value [($P_{model} > F$) < 0.0001] (Table 4). The fitness of the model was examined by determination coefficient ($R^2$). In this case, the $R^2$ value of 0.9896 indicated that 98.96% of the total variability in the response could be explained by this model. A regression model with $R^2 > 0.9$ was considered as having a very high correlation (Chen et al., 2009a). Therefore, the present $R^2$-value reflected a very good fit between the observed and predicted responses, and implied that the model is reliable for predicting inulinase recovery. The value of the adjusted determination coefficient (Adj $R^2 = 0.9803$) confirmed the significance of the model as well. Among model terms, $X_1$, $X_2$, $X_1^2$, $X_2^2$ and $X_3^2$ were very significant with a probability of 99%, while $X_3$ with a probability of 95% (Table 4).

The response of inulinase recovery to solid/liquid ratio, stirring rate and temperature for the above regression model was plotted in Figs. 1–3. The optimal levels for the maximum inulinase recov-

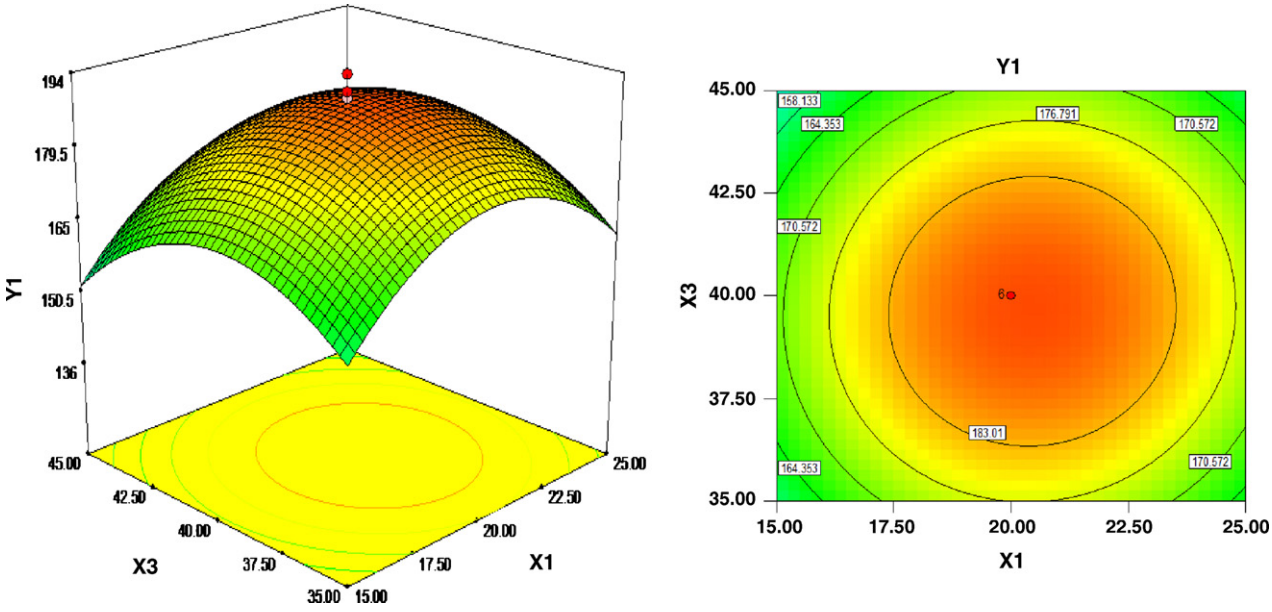


**Fig. 2.** Effects of solid/liquid ratio ($X_1$) and temperature ($X_3$) on the recovery of inulinase ($Y_1$).

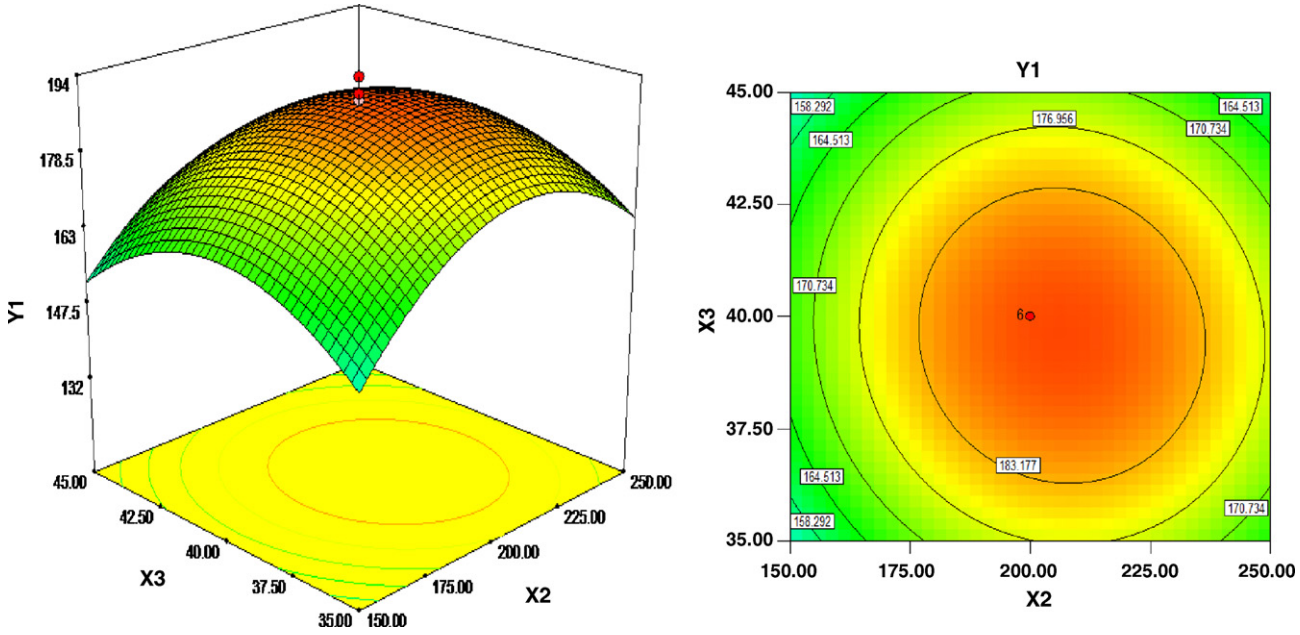


**Fig. 3.** Effects of stirring rate ($X_2$) and temperature ($X_3$) on the recovery of inulinase ($Y_1$).

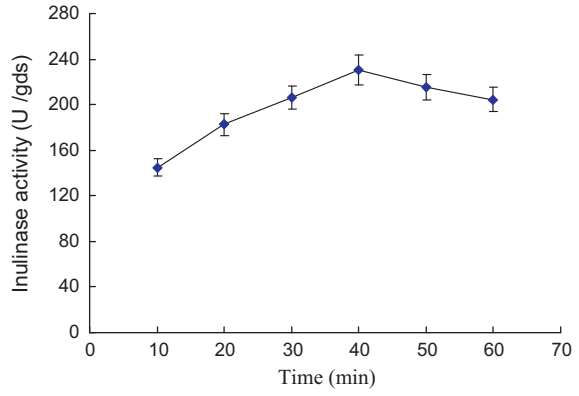


**Fig. 4.** Effects of extraction time on the recovery of inulinase. Data are given as means ± SD, $n = 3$.

ery based on the model were calculated and the value was 1:20.5, 207 rpm and 39.6 °C for solid/liquid ratio, stirring rate and temperature, respectively.

### 3.3. Effect of extraction time on inulinase recovery

Under the optimal extraction conditions obtained, the effect of different extraction time on inulinase recovery was studied. As shown in Fig. 4, the maximum recovery of inulinase was achieved after 40 min.

## 4. Conclusions

In this study, the effects of the type of solvent, solid/liquid ratio, stirring rate, temperature, and extraction time on recovery of inulinase from solid state fermentation of *A. ficuum* JNSP5-06 were investigated. The extraction parameters for inulinase recovery were optimized by central composite rotatable design. The maximum recovery of inulinase was obtained using 0.1 M sodium acetate buffer of pH 4.5 as extraction solvent, in a solid/liquid ratio of 1:20.5, at 39.6 °C and 207 rpm for 40 min.

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