# Optimal immobilization of β-glucosidase into chitosan beads using response surface methodology

**Ying Zhou[1,2]** · **Lufeng Wang[1]** ✉ · **Ting Wu[1]** · **Xixuan Tang[1]** · **Siyi Pan[1]**

1 Huazhong Agricultural University, College of Food Science and Technology, Hongshan District, Wuhan, China

2 Wuhan Grain Administration, Wuhan, Hubei, China

✉ Corresponding author: wanglf@mail.hzau.edu.cn

## Abstract

**Background:** β-Glucosidase is known as an effective catalyst for the hydrolysis of various glycosides and immobilization is one of the most efficient strategies to improve its activity recovery and properties.
**Results:** Crosslinking-adsorption-crosslinking method was employed to immobilize β-glucosidase into chitosan beads and response surface methodology (RSM) was used to optimize the immobilized conditions of the maximum activity recovery. Enzyme concentration and adsorption time were found to be significant influence factors, and the maximum activity recovery (50.75%) obtained from response surface methodology was in excellent agreement with experimental value (50.81%). Furthermore, various characteristics of immobilized β-glucosidase were evaluated. Compared to the free β-glucosidase, the immobilized enzyme exhibited broader pH and temperature ranges, enhanced thermal stability, better storage stability and reusability and higher accessibility of the substrate to the immobilized β-glucosidase.
**Conclusion:** Response surface methodology (RSM) was proved to be much economical for optimum immobilization of β-glucosidase into chitosan beads.

**Keywords:** β-glucosidase; characters; chitosan beads; immobilization; response surface methodology.

## INTRODUCTION

β-glucosidase (EC 3.2.1.21), widely existed in various types of living organisms, is known as an effective catalyst for the hydrolysis of various glycosides (Njokweni et al. 2012). Recently, it was reported to be used for enhancement of aroma in wine (Palmeri and Spagna, 2007), tea (Su et al. 2010), fruit (Fan et al. 2011), hydrolysis of cellulose (Shewale, 1982), and bioconversion of isoflavone glycosides to aglycones (Rekha and Vijayalakshmi, 2010). It is currently receiving much attention for their potential industrial applications. However, there are still some limitations for the widely application of β-glucosidase, because of its structure instability and the high cost of recovery of enzymes for repeated applications (Nisha et al. 2012; Dicosimo et al. 2013; Sheldon and van Pelt, 2013). Immobilization of β-glucosidase is considered to be the promising method to overcome these drawbacks.

Immobilized enzymes are versatile catalysts in the laboratory and in an industrial scale. Immobilization facilitates the efficient recovery and reuse of costly enzymes (Sheldon, 2007). Additional advantages include enhanced stability, great operational control, flexibility of reactor design, and easy separation from catalyst and unreacted substrate contamination. Chitosan is an inexpensive, inert, hydrophilic, biocompatible support, and it is considered to be an excellent carrier for enzyme immobilization (Çetinus and Öztop, 2003; Krajewska, 2004). The presence of amino groups of chitosan facilitates covalent binding of enzymes. Immobilization can be carried out either by entrapment into chitosan

beads (Freeman and Dror, 1994) or by covalent binding to transparent chitosan films (Çetinus and Öztop, 2000) or by using glutaraldehyde from the formation of the Schiff's base (Ravi Kumar, 1999). β-glucosidase has been reported to be immobilized in chitosan gels, but the maximum activity recovery of immobilization was only 26.9% (Spagna et al. 2002). So it is of great importance to further improve the activity recovery of immobilized β-glucosidase into chitosan beads for industrial application.

The designing of an efficient immobilized enzyme is a multivariate process involving many factors that could affect immobilization efficiency. Response surface methodology (RSM) is a statistically designed experimental protocol for developing, improving, and optimizing processes (Bouaid et al. 2009). It helps to identify the effect of the interactions of different design variables on the response when they are varied simultaneously. Furthermore, central composite design (CCD), the most successful factorial design, was also used for the optimization of parameters with a limited number of experiments and minimum costs (Singh et al. 2008). Recently, RSM has been successfully used for the optimization of several immobilization processes, including immobilization of *Gluconobacter oxydans* in Ca-alginate gel (Wu et al. 2010), *Thermomyces lanuginosus* lipase on styrene-divinylbenzene copolymer (Aybastier and Demir, 2010), acetylcholine esterase on ceramic packing (Ebrahimi et al. 2010) and chick pea β-galactosidase to alkylamine glass (Kishore and Kayastha, 2012).

In this research, β-glucosidase was immobilized into chitosan beads and response surface methodology (RSM) was used to optimize the immobilized conditions of β-glucosidase for the maximum activity recovery. Furthermore, various properties for free and immobilized β-glucosidase such as the Ph-activity curve, the temperature-activity curve, thermal stability, storage stability and reusability were evaluated.

## MATERIALS AND METHODS

β-Glucosidase (2.18 U/mg) was purchased from Sigma-Aldrich Co. LLC. p-NPG was provided by Shanghai Source Leaves Biological Technology Co., LTD. Chitosan (Degree of deacetylation 90%) was obtained from Wuhan Huashun Biological Technology Co., LTD. Glutaraldehyde (GA) was bought from Sinopharm Chemical Reagent Co., Ltd. All other chemicals were of analytical grade.

### Preparation of pretreated and crosslinking chitosan beads

The pretreated chitosan beads were prepared based on a reported method with minor modification (Çetinus and Öztop, 2003; Hung et al. 2003). Chitosan flakes (2.0 g) were dissolved in 100 mL of acetic acid solution (5.0%, *v/v*) by needles (0.7 x 30 TWLB). The resulting solution was added into 200 mL of NaOH solution (2.0 M) containing 40 mL of ethanol through a needle with a diameter of 0.7 mm at room temperature, and the chitosan gelled spheres formed instantaneously. After hardening, the pretreated chitosan beads were separated and washed with deionized water until the filtrate became neutral (pH 7.0). The diameter of the pretreated beads was approximately 1.5-1.8 mm.

Then the pretreated chitosan beads (0.1 g) were added into 10 mL of glutaraldehyde solution (3.0%) and the mixtures were stirred at 25ºC and 150 rpm for 2 hrs. The crosslinking chitosan beads were separated on a filter and washed with 100 mM phosphate-citric acid buffer (pH 5.0).

### Immobilization of β-glucosidase into chitosan beads

**Direct adsorption.** 0.1 g pretreated chitosan beads were added into 1 mL β-glucosidase solution (0.1 mg/mL) and the solution was stirred at 25ºC, 150 rpm for 5.0 hrs. Then the chitosan beads with adsorbed enzyme were separated and washed thrice with 100 mM phosphate-citric acid buffer (pH 5.0) to remove unbound enzymes.

**Adsorption-crosslinking.** The procedure of adsorption was the same as the above description. After direct adsorption, the washed chitosan beads with adsorbed enzyme were added into 10 mL of 3.0% (*v/v*) glutaraldehyde solution. The mixture was stirred at 25ºC and 150 rpm for 2.0 hrs. The immobilized enzyme was separated and washed thrice with 100 mM phosphate-citric acid buffer (pH 5.0).

**Crosslinking-adsorption.** 0.1 g crosslinking chitosan beads and 1 mL β-glucosidase solution (0.1 mg/ml) were mixed and stirred at 25ºC, 150 rpm for 5.0 hrs. The crosslinking chitosan beads with adsorbed enzyme were washed thrice with 100 mM phosphate-citric acid buffer (pH 5.0) to remove unbound enzymes.

**Crosslinking-adsorption-crosslinking.** The main procedure was the combination of crosslinking-adsorption and adsorption-crosslinking.

### Determination the activity of β-glucosidase

The activity of β-glucosidase was determined by monitoring the release of p-NPG. A total of 100 µL of enzyme solution was incubated with 900 µL of 100 mM phosphate-citric acid buffer (pH 5.0) containing 10 mM p-NPG for 15 min at 50ºC. The amount of p-nitrophenol released was measured at $\lambda = 400$ nm after addition of 2 M $Na_2CO_3$ to the reaction mixtures. One unit of p-NPG-hydrolyzing activity was defined as the amount of enzyme equivalent to release 1 µmol of p-nitrophenol per minute.

The activity of immobilized β-glucosidase was measured by the same method as above, except that 100 µL of the enzyme solution was replaced by 100 µL phosphate-citric acid buffer and a given amount of immobilized enzyme.

The immobilized β-glucosidase activity recovery was calculated as: activity recovery (%) = (total activity of immobilized enzyme/total activity of enzyme solution) x 100%.

### Experimental design and statistical analyses

Factorial design was performed using Minitab Statistical Software (Minitab 15) to screen the significant influence factors. The influence factors were chosen after a series of preliminary experiments (data not shown). Effects of various factors on immobilization of β-glucosidase into chitosan beads were evaluated using 2-level-10-factor experimental design with 16 experiments.

Response surface methodology was used to determine optimum conditions of immobilization to acquire the maximum activity recovery, and a 3-level-2-factor central composite design with 14 experiments was performed. All experiments were repeated at least three times. Minitab Statistical Software was used both in experimental design and the statistical analysis of the results.

### Steady state kinetics

The optimum pH of free and immobilized β-glucosidase was determined in 100 mM $Na_2HPO_4$-$C_6H_8O_7$ buffer (pH 3.0-7.0). The optimum temperature of free and immobilized β-glucosidase was determined by assaying the enzyme activity at various temperatures (30-80ºC) in 100 mM phosphate-citric acid buffer (pH 5.0). The thermal stability of free and immobilized β-glucosidase was evaluated as follow: The β-glucosidase was kept in a water bath for 1 hr at different temperatures (30-80ºC), and followed by residual activity assay under standard conditions.

The Michaelis constant (*Km*) and maximum velocity (*Vmax*) of free and immobilized β-glucosidase were measured by assaying the enzyme activity at different p-NPG concentrations. The *Km* and *Vmax* were calculated using Lineweaver-Burk plot (Lineweaver and Burk, 1934).

### Evaluation of the storage stability and reusability

The activity of free and immobilized β-glucosidase were evaluated after a long period (50 days) storage in phosphate-citric acid buffer (pH 5.0) at 4ºC under different time intervals.

For reusability assessment, stored immobilized β-glucosidase was reused 10 times and the residual activity was measured. After each reaction, the immobilized sample was washed with 100 mM $Na_2HPO_4$-$C_6H_8O_7$ buffer (pH 5.0) to remove any attached substrate. All conditions were kept constant for every batch.

## RESULTS AND DISCUSSION

### Effects of immobilizing methods

Enzymes can be immobilized by various methods, including physical adsorption, entrapment and covalent binding (Wang et al. 2008; Tran and Balkus, 2011; Dicosimo et al. 2013; Sheldon and van Pelt, 2013). Covalent bonding methods have been widely studied because of the high bonding strength that occurs between the enzyme and the solid support (Park et al. 2001; Soares et al. 2003; Lee et al. 2007).

In present work, four different covalent binding methods were used to immobilize β-glucosidase, and the results of the activity recovery are listed in Table 1. According to these results, the crosslinking-adsorption-crosslinking method was the best one (Entry 4), and the activity recovery reached 32.55%. The loss of activation may be caused by the restriction of the support which resulted in limited mobility and accessibility of the active sites (Fan et al. 2002). Consequently, the crosslinking-adsorption-crosslinking method was chosen to immobilize β-glucosidase in the following experiments.

**Table 1. Effects of different immobilizing methods on the activity recovery of immobilized β-glucosidase.**

| Entry | Immobilizing method | Activity recovery (%) |
|:-----:|:-------------------|:---------------------:|
| 1 | Direct adsorption | 6.14 ± 0.0002 |
| 2 | Adsorption-crosslinking | 15.88 ± 0.0005 |
| 3 | Crosslinking-adsorption | 28.91 ± 0.0007 |
| **4** | **Crosslinking-adsorption-crosslinking** | **32.55** ± 0.0004 |

### Optimization of immobilized conditions

**Screening of the significant factors by factorial design.** To further improve the activity recovery of immobilized β-glucosidase, a 2-level-10-factor factorial design was firstly employed to screen the significant factors for immobilization of β-glucosidase, and the response was set as the activity recovery of immobilized β-glucosidase (Table 2). Table 3 shows their analysis of variance and model-fitting results. Among those factors, enzyme concentration and adsorption time were significant factors (P value < 0.05, entries 7 and 8).

**Optimization of immobilized conditions by response surface methodology.** Based on the analysis of the factorial design, enzyme concentration and adsorption time significantly affected the immobilization of β-glucosidase into chitosan beads. Thus, these two factors were further optimized using response surface methodology to obtain the maximum activity recovery of immobilized β-glucosidase. To assess the influence of each factor and their interactions, a central composite design of 6 centre points with 14 experiments was performed. The levels of each factor are given in Table 4 and the experimental data on the basis of activity recovery are recorded in Table 5.

By regression analysis (Table 6), a quadratic polynomial equation was established to explain the relationship between the activity recovery and independent variables as follows:

$$Y = 0.430546 - 0.10608 \times A + 0.049386 \times B - 0.04129 \times A^2 - 0.01102 \times A \times B - 0.11533 \times B^2$$

Where Y, A and B represent the activity recovery, enzyme concentration and adsorption time, respectively. Regression analysis (Table 6) showed a coefficient of determination ($R^2$) value of 0.9996, indicating that this model could explain 99.96% of the variability in activity recovery. The adjusted $R^2$ value (0.9992) was also high, meaning that this model has high significance. The predicted $R^2$ value (0.9970) was considered reasonable to predict responses by using the model. The P-value serves as a tool for checking the significance of each coefficient. A smaller P-value indicates a higher level of significance for the corresponding coefficient (Li et al. 2007). From regression analysis (Table 6), it can

be also observed that the regression coefficients of the linear terms (A, B), the quadratic terms ($A^2$, $B^2$) and the interaction term (A x B) were all significant at 1% level, implying that above coefficients have noticeable effects on the immobilization of β-glucosidase.

**Table 2. Factorial design (2^10) matrix and experimental results of activity recovery.**

| Run | A[a] | B[b] | C[c] | D[d] | E[e] | F[f] | G[g] | H[h] | I[i] | J[j] | Activity recovery (%) |
|-----|----|----|----|----|-----|-----|----|------|----|----|----------------------|
| 1 | 4 | 1 | 3 | 20 | 5.4 | 0.3 | 7 | 0.05 | 1 | 30 | 19.79 ± 0.0811 |
| 2 | 6 | 3 | 1 | 30 | 4.6 | 0.1 | 3 | 0.1 | 1 | 30 | 25.39 ± 0.1804 |
| 3 | 6 | 1 | 1 | 20 | 5.4 | 0.1 | 7 | 0.1 | 1 | 20 | 31.08 ± 0.0180 |
| 4 | 6 | 3 | 3 | 20 | 5.4 | 0.1 | 3 | 0.05 | 1 | 30 | 24.93 ± 0.0494 |
| 5 | 4 | 1 | 1 | 20 | 4.6 | 0.1 | 3 | 0.05 | 3 | 30 | 21.55 ± 0.0665 |
| 6 | 6 | 3 | 3 | 30 | 5.4 | 0.3 | 7 | 0.1 | 3 | 30 | 16.12 ± 0.0485 |
| 7 | 4 | 1 | 3 | 30 | 5.4 | 0.1 | 3 | 0.1 | 3 | 30 | 30.52 ± 0.0320 |
| 8 | 6 | 1 | 3 | 30 | 4.6 | 0.1 | 7 | 0.05 | 1 | 20 | 32.90 ± 0.2151 |
| 9 | 6 | 3 | 1 | 20 | 4.6 | 0.3 | 7 | 0.05 | 3 | 30 | 18.84 ± 0.0200 |
| **10** | **4** | **3** | **1** | **30** | **5.4** | **0.1** | **7** | **0.05** | **3** | **20** | **39.95** ± 0.0722 |
| 11 | 4 | 1 | 1 | 30 | 4.6 | 0.3 | 7 | 0.1 | 1 | 30 | 19.58 ± 0.0488 |
| 12 | 6 | 1 | 1 | 30 | 5.4 | 0.3 | 3 | 0.05 | 3 | 20 | 13.59 ± 0.0841 |
| 13 | 4 | 3 | 1 | 20 | 5.4 | 0.3 | 3 | 0.1 | 1 | 20 | 16.26 ± 0.1328 |
| 14 | 4 | 3 | 3 | 20 | 4.6 | 0.1 | 7 | 0.1 | 3 | 20 | 34.22 ± 0.0657 |
| 15 | 4 | 3 | 3 | 30 | 4.6 | 0.3 | 3 | 0.05 | 1 | 20 | 16.87 ± 0.0385 |
| 16 | 6 | 1 | 3 | 20 | 4.6 | 0.3 | 3 | 0.1 | 3 | 20 | 17.44 ± 0.0176 |

[a] A: NaOH/ethanol ratio.
[b] B: GA concentration for the pre-crosslinking (%).
[c] C: the pre-crosslinking time (h).
[d] D: the pre-crosslinking temperature (ºC).
[e] E: pH.
[f] F: enzyme concentration (mg/ml).
[g] G: adsorption time (h).
[h] H: GA concentration for the re-crosslinking (%).
[i] I: the re-crosslinking time (h).
[j] J: the re-crosslinking temperature (ºC).

**Table 3. Analysis of variance and model-fitting results for the 10-variable factorial design matrix.**

| Term | Effect | Coefficient | SE coefficient | T ratio | P value |
|------|--------|-------------|----------------|---------|---------|
| Constant | Term | 0.2369 | 0.009 | 26.32 | 0.000[a] |
| A | -0.02307 | -0.01154 | 0.009 | -1.28 | 0.256 |
| B | 0.00767 | 0.00383 | 0.009 | 0.43 | 0.688 |
| C | 0.00817 | 0.00409 | 0.009 | 0.45 | 0.669 |
| D | 0.01352 | 0.00676 | 0.009 | 0.75 | 0.486 |
| E | 0.00683 | 0.00342 | 0.009 | 0.38 | 0.720 |
| F | -0.12756 | -0.06378 | 0.009 | -7.09 | 0.001[a] |
| G | 0.05743 | 0.02871 | 0.009 | 3.19 | 0.024[b] |
| H | 0.00273 | 0.00137 | 0.009 | 0.15 | 0.885 |
| I | 0.00679 | 0.00339 | 0.009 | 0.38 | 0.722 |
| J | -0.03197 | -0.01599 | 0.009 | -1.78 | 0.136 |

[a] Significant at 1% level.
[b] Significant at 5% level.

The summary of analysis of variance (ANOVA) for the model of activity recovery is given in Table 7. The calculated F ratio was 3233.61 and P value was 0.000, implying that the model is highly significant and satisfying. The large value of F indicates that the most of the variation in the response can be explained by the regression equation. The associated P value was used to estimate whether F was large enough to indicate statistical significance. If P value was lower than 0.05, it indicates that the model was statistically significant (Ranjan et al. 2009). Moreover, the lack of fit F ratio was 1.98 and P value was 0.259, indicating that the lack of fit was also statistically significant.

**Table 4. Levels of design variables.** Coding of levels (α = 1.414).

| Design variable | Coding of levels | | | | |
|---|---|---|---|---|---|
| | -α | -1 | 0 | +1 | +α |
| Enzyme concentration (mg/mL) Adsorption time (hrs) | 0.059 2.18 | 0.13 | 0.25 | 0.37 | 0.341 7.82 |

**Table 5. Response surface central composite design of factors and activity recovery of different enzyme concentration (A) at different adsorption times (B).**

| Run | Coded levels | | Actual levels | | Activity recovery (%) |
|---|---|---|---|---|---|
| | A | B | A | B | |
| 1 | 1 | -1 | 0.3 | 3 | 12.43 ± 0.0755 |
| 2 | 0 | 0 | 0.2 | 5 | 42.20 ± 0.0836 |
| 3 | 0 | 0 | 0.2 | 5 | 42.05 ± 0.1430 |
| 4 | -1 | -1 | 0.1 | 3 | 31.02 ± 0.0733 |
| 5 | 1 | 1 | 0.3 | 7 | 20.38 ± 0.0840 |
| 6 | 0 | 0 | 0.2 | 5 | 42.78 ± 0.0954 |
| 7 | -1 | 1 | 0.1 | 7 | 43.38 ± 0.0330 |
| 8 | 0 | -1.41 | 0.2 | 2.18 | 13.80 ± 0.0371 |
| 9 | 0 | 0 | 0.2 | 5 | 43.54 ± 0.0817 |
| 10 | 0 | 0 | 0.2 | 5 | 43.93 ± 0.0673 |
| 11 | 0 | 1.41 | 0.2 | 7.82 | 27.37 ± 0.0431 |
| **12** | **-1.41** | **0** | **0.06** | **5** | **50.69** ± 0.0320 |
| 13 | 1.41 | 0 | 0.34 | 5 | 20.09 ± 0.0384 |
| 14 | 0 | 0 | 0.2 | 5 | 43.82 ± 0.0834 |

**Table 6. Estimated regression coefficients and R-squared values of the central composite design.**

| Term | Coefficient | SE coefficient | T ratio | P value |
|---|---|---|---|---|
| Constant | 0.430546 | 0.001492 | 288.509 | 0.000[a] |
| A: enzyme concentration | -0.10608 | 0.001292 | -82.08 | 0.000[a] |
| adsorption time | 0.049386 | 0.001292 | 38.213 | 0.000[a] |
| A*A | -0.04129 | 0.001345 | -30.692 | 0.000[a] |
| B*B | -0.11533 | 0.001345 | -85.733 | 0.000[a] |
| A*B | -0.01102 | 0.001828 | -6.03 | 0.001[a] |
| $R^2 = 0.9996$ | $R^2$ (adj) = 0.9992 | $R^2$ (pred) = 0.9970 | | |

[a] Significant at 1% level.

**Table 7. Analysis of variance (ANOVA) for the results of the central composite design.**

| Source | DF | Seq SS | Adj SS | Adj MS | F ratio | P value |
|---|---|---|---|---|---|---|
| Regression | 5 | 0.216038 | 0.216038 | 0.043208 | 3233.61 | 0.000[a] |
| Linear | 2 | 0.109533 | 0.109533 | 0.054767 | 4098.68 | 0.000 |
| Square | 2 | 0.106019 | 0.106019 | 0.053009 | 3967.17 | 0.000 |
| Interaction | 1 | 0.000486 | 0.000486 | 0.000486 | 36.36 | 0.001 |
| Residual error | 7 | 0.000094 | 0.000094 | 0.000013 | | |
| Lack of fit | 3 | 0.000056 | 0.000056 | 0.000019 | 1.98 | 0.259 |
| Pure error | 4 | 0.000038 | 0.000038 | 0.000009 | | |
| Total | 13 | 0.216709 | | | | |

[a] Significant at 1% level.

In order to understand the interaction between enzyme concentration and adsorption time as well as determine the maximum activity recovery corresponding to the optimum conditions of both variables, their response surface and contour plots are represented in Figure 1 and Figure 2, which indicates the response surface and contour plots have the same results. Optimal conditions for enzyme concentration and adsorption time were 67.15 μg/mL and 5.54 hrs, respectively, leading to the maximum activity recovery of 50.75%. A verification experiment was performed under the optimal conditions to validate the reliability and accuracy of the model. The experimental activity recovery was found to be 50.81%, which is in excellent agreement with the value predicted by the model based on central composite design (50.75%). Consequently, this model could be considered reliable and accurate for predicting the activity recovery of immobilization of β-glucosidase. The activity recovery of immobilized β-glucosidase on chitosan in previous report was only 26.90% (Spagna et al. 2002). Evidently, the activity recovery of immobilized β-glucosidase on chitosan beads has been obviously improved in our study. Furthermore, the activity recovery in our study is much higher than that of immobilized β-glucosidase on other various carriers, such as sodium alginate (28.20%) (Fan et al. 2011), S-layer (37.00%) (Balogh et al. 2005), Eupergit C (30.00%) (Tu et al. 2006), and sol-gel beads (28.00%) (O'Neill et al. 2002).

**Steady state kinetics**

**Optimum pH.** The optimal pH of free and immobilized β-glucosidase was investigated at diverse pH (3.0-7.0) at the same temperature. As shown in Figure 3, the optimal pH of free and immobilized β-glucosidase were both 5.0, but the immobilized β-glucosidase has a broader pH range of high activity.

**Optimum temperature and thermal stability.** The optimal temperature of free and immobilized β-glucosidase was investigated in the temperatures range of 30-80ºC. Figure 4 shows that the optimal temperature is 60ºC for the free β-glucosidase and 70ºC for the immobilized β-glucosidase. This shift toward high temperature for the immobilized β-glucosidase was similar with the published literatures (Yodoya et al. 2003; Jung et al. 2012). Moreover, Immobilization β-glucosidase evidently widened the working temperature range and the relative activity of the immobilized β-glucosidase still remained over 84% at 40-70ºC. The reason for increased temperature stability maybe because that immobilization of β-glucosidase increased the rigidity of the enzyme and the support had a protection effect at the high temperatures at which enzyme deactivation took place (Yodoya et al. 2003; Singh et al. 2011).

The thermal stability of free and immobilized β-glucosidase was investigated after incubating for 1 hr at diverse temperatures (30-80ºC). As shown in Figure 5, the activity of the immobilized β-glucosidase decreased much more slowly than that of the free β-glucosidase at the temperature range from 50ºC to 60ºC. The free enzyme only retained 35% of its initial activity, whereas the immobilized enzyme was found to keep almost 64% of its initial activity. Since the immobilization increased the rigidity of the enzyme, the heat-resistance of immobilized β-glucosidase increased considerably (Chiou and Wu, 2004; Dwevedi and Kayastha, 2009). Thus, compared to the original free enzyme, the immobilized enzyme could work in harsh environmental conditions with less activity loss (Spagna et al. 2002; Reddy and Kayastha, 2006; Dwevedi and Kayastha, 2009).

## Kinetic parameters

As expected, the $K_m$ and $V_{max}$ values of β-glucosidase were significantly affected after immobilization into chitosan beads. As shown in Figure 6, Kinetic parameters, $K_m$ values of the free and immobilized β-glucosidase were found to be 3.12 and 2.51 mM, respectively. The decrease in $K_m$ of immobilized β-glucosidase may be due to the affinity change of the enzyme to its substrate or conformational change in the immobilization of β-glucosidase into chitosan beads, which caused higher accessibility of the substrate to the immobilized β-glucosidase. Similar results were also observed in β-glucosidase immobilization on alginate (Su et al. 2010; Fan et al. 2011).

## Storage stability and reusability

The storage stability of an enzyme is a prior condition to evaluate its industrial applicability. The free β-glucosidase lost more than 80% of its initial activity after storage for 50 days, whereas the immobilized β-glucosidase was found to lose less than 20% of its initial activity (Figure 7a). The results showed that the immobilized β-glucosidase exhibited higher stability than the free one, and similar phenomenon has also been documented recently (Su et al. 2010; Fan et al. 2011; González-Pombo et al. 2011).

The reusability of immobilized β-glucosidase is of key importance for their industrial application (Gupta et al. 2009; Zhang et al. 2010). As indicated in Figure 7b, the activity of immobilized enzymes was retained more than 89% after 10 reuses. Evidently, the immobilized β-glucosidase has a more stable performance in reuse. The activity loss of immobilized enzyme upon reuse could be caused by the weakening of binding strength between the enzyme and supporter. Moreover, the immobilized enzyme might be distorted by the frequent encountering between the active site and substrate, and thereby its catalytic efficiency might be lowered (Dwevedi and Kayastha, 2009). Similar results about reusability for the immobilized β-glucosidase were reported in other immobilization studies (O'Neill et al. 2002; Singh et al. 2011).

## CONCLUDING REMARKS

This is the first report about optimization of β-glucosidase immobilization into chitosan beads by response surface methodology. After immobilized by crosslinking-adsorption-crosslinking method, a factorial design was applied to successfully select two significant factors including enzyme concentration and adsorption time, and then RSM was employed to obtain the optimal levels of enzyme concentration (67.15 μg/ml) and adsorption time (5.54 hrs). The predicted maximum activity recovery (50.75%) is in excellent agreement with the experimental value (50.81%), which is much higher than that of other immobilized methods reported before. The immobilized β-glucosidase showed improved enzymatic properties than the free form, such as broader pH and temperature ranges, enhanced thermal stability, higher accessibility of the substrate, better storage stability and superior reusability. Improved activity recovery and enzymatic properties of immobilized β-glucosidase in present study will promote its industrial application.

## REFERENCES

AYBASTIER, Ö. and DEMIR, C. (2010). Optimization of immobilization conditions of *Thermomyces lanuginosus* lipase on styrene-divinylbenzene copolymer using response surface methodology. *Journal of Molecular Catalysis B: Enzymatic*, vol. 63, no. 3-4, p. 170-178. **[CrossRef]**

BALOGH, T.; TÓTH, A. and KOSÁRY, J. (2005). S-layer, a new support for enzyme immobilization. *Acta Alimentaria*, vol. 34, no. 2, p. 203-208. **[CrossRef]**

BOUAID, A.; MARTÍNEZ, M. and ARACIL, J. (2009). Production of biodiesel from bioethanol and *Brassica carinata* oil: Oxidation stability study. *Bioresource Technology*, vol. 100, no. 7, p. 2234-2239. **[CrossRef]**

ÇETINUS, S.A. and ÖZTOP, H.N. (2000). Immobilization of catalase on chitosan film. *Enzyme and Microbial Technology*, vol. 26, no. 7, p. 497-501. **[CrossRef]**

ÇETINUS, S.A. and ÖZTOP, H.N. (2003). Immobilization of catalase into chemically crosslinked chitosan beads. *Enzyme and Microbial Technology*, vol. 32, no. 7, p. 889-894. **[CrossRef]**

CHIOU, S.H. and WU, W.T. (2004). Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. *Biomaterials*, vol. 25, no. 2, p. 197-204. **[CrossRef]**

DICOSIMO, R.; MCAULIFFE, J.; POULOSEB, A.J. and BOHLMANN, G. (2013). Industrial use of immobilized enzymes. *Chemical Society Reviews*, vol. 42, no. 15, p. 6437-6474. **[CrossRef]**

DWEVEDI, A. and KAYASTHA, A.M. (2009). Optimal immobilization of *β*-galactosidase from Pea (*Ps*BGAL) onto Sephadex and chitosan beads using response surface methodology and its applications. *Bioresource Technology*, vol. 100, no. 10, p. 2667-2675. **[CrossRef]**

EBRAHIMI, B.; SHOJAOSADATI, S.A.; RANAIE, S.O. and MOUSAVI, S.M. (2010). Optimization and evaluation of acetylcholine esterase immobilization on ceramic packing using response surface methodology. *Process Biochemistry*, vol. 45, no. 1, p. 81-87. **[CrossRef]**

FAN, Q.H.; LI, Y.M. and CHAN, A.S.C. (2002). Recoverable catalysts for asymmetric organic synthesis. *Chemical Reviews,* vol. 102, no. 10, p. 3385-3466. **[CrossRef]**

FAN, G.; XU, Y.; ZHANG, X.; LEI, S.; YANG, S. and PAN, S. (2011). Characteristics of immobilised β-glucosidase and its effect on bound volatile compounds in orange juice. *International Journal of Food Science & Technology*, vol. 46, no. 11, p. 2312-2320. **[CrossRef]**

FREEMAN, A. and DROR, Y. (1994). Immobilization of "disguised" yeast in chemically crosslinked chitosan beads. *Biotechnology and Bioengineering*, vol. 44, no. 9, p. 1083-1088. **[CrossRef]**

GONZÁLEZ-POMBO, P.; FARIÑA, L.; CARRAU, F.; BATISTA-VIERA, F. and BRENA, B.M. (2011). A novel extracellular β-glucosidase from *Issatchenkia terricola*: Isolation, immobilization and application for aroma enhancement of white Muscat wine. *Process Biochemistry*, vol. 46, no. 1, p. 385-389. **[CrossRef]**

GUPTA, P.; DUTT, K.; MISRA, S.; RAGHUWANSHI, S. and SAXENA, R.K. (2009). Characterization of cross-linked immobilized lipase from thermophilic mould *Thermomyces lanuginosa* using glutaraldehyde. *Bioresource Technology*, vol. 100, no. 18, p. 4074-4076. **[CrossRef]**

HUNG, T.C.; GIRIDHAR, R.; CHIOU, S.H. and WU, W.T. (2003). Binary immobilization of *Candida rugosa* lipase on chitosan. *Journal of Molecular Catalysis B: Enzymatic*, vol. 26, no. 1-2, p. 69-78. **[CrossRef]**

JUNG, Y.R.; SHIN, H.Y.; SONG, Y.S.; KIM, S.B. and KIM, S.W. (2012). Enhancement of immobilized enzyme activity by pretreatment of *β*-glucosidase with cellobiose and glucose. *Journal of Industrial and Engineering Chemistry*, vol. 18, no. 2, p. 702-706. **[CrossRef]**

KISHORE, D. and KAYASTHA, A.M. (2012). Optimisation of immobilisation conditions for chick pea β-galactosidase (*Cp*GAL) to alkylamine glass using response surface methodology and its applications in lactose hydrolysis. *Food Chemistry*, vol. 134, no. 3, p. 1650-1657. **[CrossRef]**

KRAJEWSKA, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: A review. *Enzyme and Microbial Technology*, vol. 35, no. 2-3, p. 126-139. **[CrossRef]**

LEE, D.H.; KIM, J.M.; SHIN, H.Y. and KIM, S.W. (2007). Optimization of lipase pretreatment prior to lipase immobilization to prevent loss of activity. *Journal of Microbiology and Biotechnology*, vol. 17, no. 4, p. 650-654.

LI, Y.; LIU, Z.; ZHAO, H.; XU, Y. and CUI, F. (2007). Statistical optimization of xylanase production from new isolated *Penicillium oxalicum* ZH-30 in submerged fermentation. *Biochemical Engineering Journal*, vol. 34, no. 1, p. 82-86. **[CrossRef]**

LINEWEAVER, H. and BURK, D. (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society*, vol. 56, no. 3, p. 658-666. **[CrossRef]**

NISHA, S.; ARUN KARTHICK, S. and GOBI, N. (2012). A review on methods, application and properties of immobilized enzyme. *Chemical Science Review and Letters*, vol. 1, no. 3, p. 148-155.

NJOKWENI, A.P.; ROSE, S.H. and VAN ZYL, W.H. (2012). Fungal β-glucosidase expression in *Saccharomyces cerevisiae*. *Journal of Industrial Microbiology & Biotechnology*, vol. 39, no. 10, p. 1445-1452. **[CrossRef]**

O'NEILL, H.; ANGLEY, C.V.; HEMERY, I.; EVANS, B.R.; DAI, S. and WOODWARD, J. (2002). Properties of carbohydrate-metabolizing enzymes immobilized in sol-gel beads: Stabilization of invertase and β-glucosidase by Blue Dextran. *Biotechnology Letters*, vol. 24, no. 10, p. 783-790. **[CrossRef]**

PALMERI, R. and SPAGNA, G. (2007). β-Glucosidase in cellular and acellular form for winemaking application. *Enzyme and Microbial Technology*, vol. 40, no. 3, p. 382-389. **[CrossRef]**

PARK, S.W.; KIM, Y.I.; CHUNG, K.H. and KIM, S.W. (2001). Improvement of stability of immobilized GL-7-ACA acylase through modification with glutaraldehyde. *Process Biochemistry*, vol. 37, no. 2, p. 153-163. **[CrossRef]**

RANJAN, D.; SRIVASTAVA, P.; TALAT, M. and HASAN, S.H. (2009). Biosorption of Cr(VI) from water using biomass of *Aeromonas hydrophila*: Central composite design for optimization of process variables. *Applied Biochemistry and Biotechnology*, vol. 158, no. 3, p. 524-539. **[CrossRef]**

RAVI KUMAR, M.N.V. (1999). Chitin and chitosan fibers: A review. *Bulletin of Materials Science*, vol. 22, no. 5, p. 905-915. **[CrossRef]**

REDDY, K.R.C. and KAYASTHA, A.M. (2006). Improved stability of urease upon coupling to alkylamine and arylamine glass and its analytical use. *Journal of Molecular Catalysis B: Enzymatic*, vol. 38, no. 2, p. 104-112. **[CrossRef]**

REKHA, C.R. and VIJAYALAKSHMI, G. (2010). Bioconversion of isoflavone glycosides to aglycones, mineral bioavailability and vitamin B complex in fermented soymilk by probiotic bacteria and yeast. *Journal of Applied Microbiology*, vol. 109, no. 4, p. 1198-1208. **[CrossRef]**

SHELDON, R.A. (2007). Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis & Catalysis*, vol. 349, no. 8-9, p. 1289-1307. **[CrossRef]**

SHELDON, R.A. and VAN PELT, S. (2013). Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews*, vol. 42, no. 15, p. 6223-6235. **[CrossRef]**

SHEWALE, J.G. (1982). β-glucosidase: Its role in cellulase synthesis and hydrolysis of cellulose. *International Journal of Biochemistry*, vol. 14, no. 6, p. 435-443. **[CrossRef]**

SINGH, G.; AHUJA, N.; BATISH, M.; CAPALASH, N. and SHARMA, P. (2008). Biobleaching of wheat straw-rich soda pulp with alkalophilic laccase from γ-proteobacterium JB: Optimization of process parameters using response surface methodology. *Bioresource Technology*, vol. 99, no. 16, p. 7472-7479. **[CrossRef]**

SINGH, R.K.; ZHANG, Y.W.; NGUYEN, N.P.T.; JEYA, M. and LEE, J.K. (2011). Covalent immobilization of β-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles. *Applied Microbiology and Biotechnology*, vol. 89, no. 2, p. 337-344. **[CrossRef]**

SOARES, C.M.F.; SANTANA, M.H.A.; ZANIN, G.M. and DE CASTRO, H.F. (2003). Covalent coupling method for lipase immobilization on controlled pore silica in the presence of nonenzymatic proteins. *Biotechnology Progress*, vol. 19, no. 3, p. 803-807. **[CrossRef]**

SPAGNA, G.; BARBAGALLO, R.N.; GRECO, E.; MANENTI, I. and PIFFERI, P.G. (2002). A mixture of purified glycosidases from *Aspergillus niger* for oenological application immobilised by inclusion in chitosan gels. *Enzyme and Microbial Technology*, vol. 30, no. 1, p. 80-89. **[CrossRef]**

SU, E.; XIA, T.; GAO, L.; DAI, Q. and ZHANG, Z. (2010). Immobilization of β-glucosidase and its aroma-increasing effect on tea beverage. *Food and Bioproducts Processing*, vol. 88, no. 2-3, p. 83-89. **[CrossRef]**

TRAN, D.N. and BALKUS, K.J. (2011). Perspective of recent progress in immobilization of enzymes. *ACS Catalysis*, vol. 1, no. 8, p. 956-968. **[CrossRef]**

TU, M.; ZHANG, X.; KURABI, A.; GILKES, N.; MABEE, W. and SADDLER, J. (2006). Immobilization of β-glucosidase on Eupergit C for lignocellulose hydrolysis. *Biotechnology Letters*, vol. 28, no. 3, p. 151-156. **[CrossRef]**

WANG, H.W.; KIM, I.H.; PARK, C.S. and LEE, J.H. (2008). Immobilization of α-amylase from *Bacillus licheniformis* on developed support using microbial transglutaminase. *Korean Journal of Chemical Engineering*, vol. 25, no. 4, p. 801-803. **[CrossRef]**

WU, J.; WANG, J.L.; LI, M.H.; LIN, J.P. and WEI, D.Z. (2010). Optimization of immobilization for selective oxidation of benzyl alcohol by *Gluconobacter oxydans* using response surface methodology. *Bioresource Technology*, vol. 101, no. 23, p. 8936-8941. **[CrossRef]**

YODOYA, S.; TAKAGI, T.; KUROTANI, M.; HAYASHI, T.; FURUTA, M.; OKA, M. and HAYASHI, T. (2003). Immobilization of bromelain onto porous copoly (γ-methyl-L-glutamate/L-leucine) beads. *European Polymer Journal*, vol. 39, no. 1, p. 173-180. **[CrossRef]**

ZHANG, S.; GAO, S. and GAO, G. (2010). Immobilization of β-galactosidase onto magnetic beads. *Applied Biochemistry and Biotechnology*, vol. 160, no. 5, p. 1386-1393. **[CrossRef]**
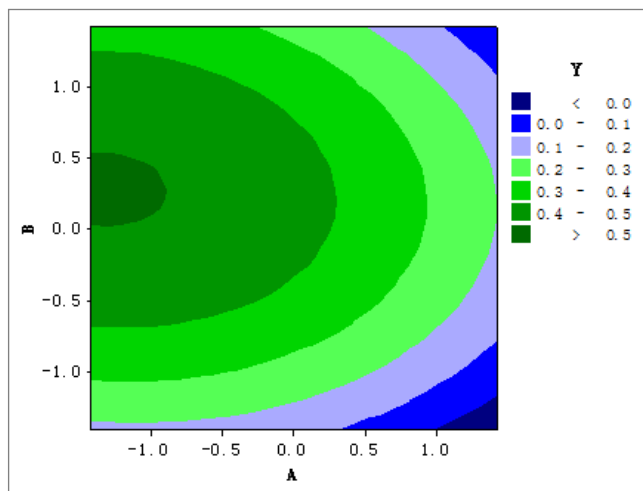
# Figures



**Fig. 1 Response surface plot showing the effect of enzyme concentration (A) and adsorption time (B) on activity recovery (Y).**
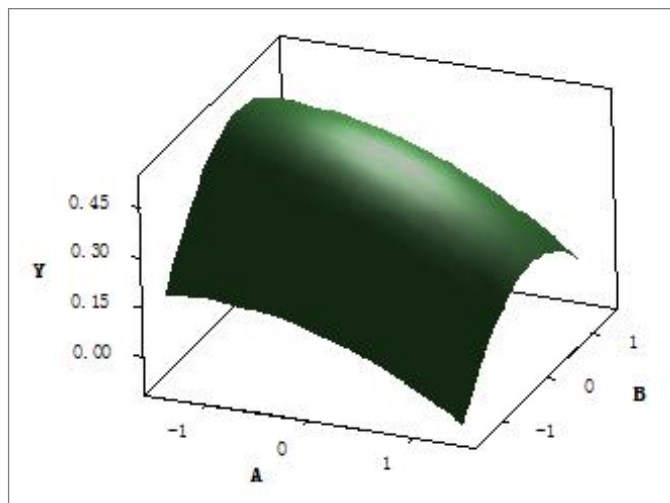


**Fig. 2 Contour plot showing activity recovery (Y) in response to varying enzyme concentration (A) and adsorption time (B).**
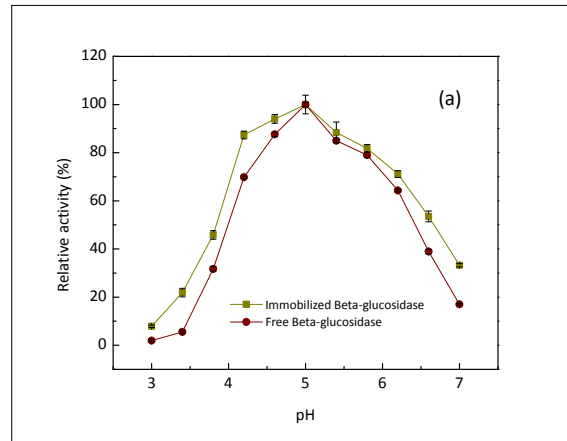
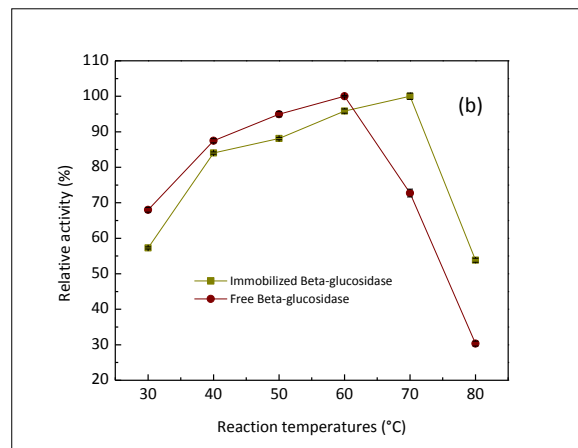**Fig. 3 Effect of pH on the activity of free and immobilized β-glucosidase.**



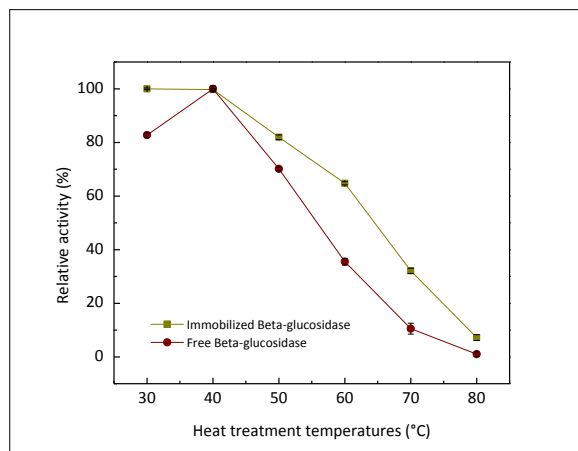**Fig. 4 Effect of temperature on the activity of free and immobilized β-glucosidase.**



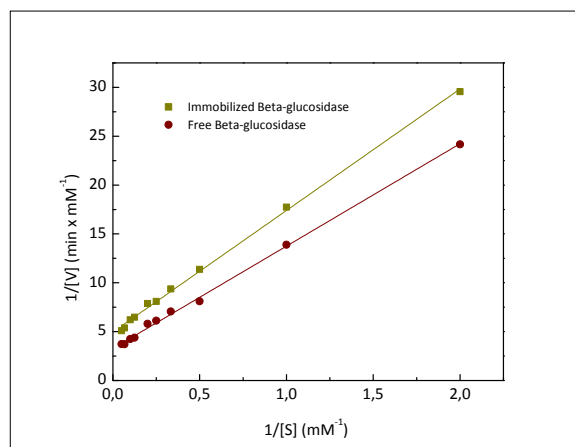**Fig. 5 Thermal stability of the free and immobilized β-glucosidase.**

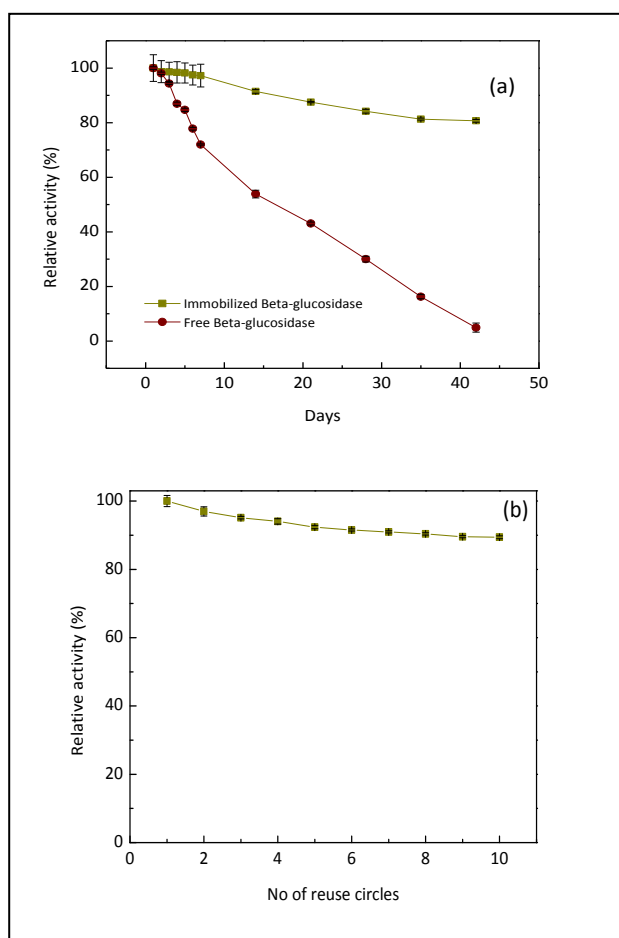**Fig. 6 Lineweaver-Burk double reciprocal plots of free and immobilized β-glucosidase.**



**Fig. 7 Storage stability (a) and reusability (b) of the free and immobilized β-glucosidase.**