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## KINETIC AND ENZYME RECYCLING STUDIES OF IMMOBILIZED $\beta$ -GLUCOSIDASE FOR LIGNOCELLULOSIC BIOMASS HYDROLYSIS

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### Abstract

$\beta$ -glucosidases are very important in the improvement of cellulose degradation rates by diminishing the inhibition of end products. Although, cellulase cost has reduced significantly in last decade but still the high costs of biomass saccharification is one of the main challenges for the commercialization of ethanol from LC biomass. In this work,  $\beta$ -glucosidase immobilization in calcium alginate was optimized using response surface methodology. 89% immobilization efficiency was achieved under optimum conditions (i.e., calcium alginate 4.19% (w/v), calcium chloride 0.14 M and  $\beta$ -glucosidase 4.42% (v/v) or 57.46 U/mL of reaction mixture). pH and temperature optima of both free and immobilized  $\beta$ -glucosidase was found to be 5 and 50 °C, respectively. Enzyme kinetics of free and immobilized  $\beta$ -glucosidase was done to evaluate Michaelis constant ( $K_m$ ) and  $V_{max}$  values, using p-nitrophenyl- $\beta$ -D-glucopyranoside (pNPG) as the substrate and glucose as inhibitor. Increase in  $K_m$  value (0.995 mM) for immobilized  $\beta$ -glucosidase as compared to the free  $\beta$ -glucosidase (0.617 mM) was observed. A decrease in  $V_{max}$  from (1191.74  $\mu\text{mol min}^{-1}$  for free  $\beta$ -glucosidase) to (736.65  $\mu\text{mol min}^{-1}$  immobilized  $\beta$ -glucosidase) was observed after immobilization. Glucose was found to inhibit  $\beta$ -glucosidase by competitive mode of inhibition ( $K_i = 3.01$  mM). Studies were carried out in a batch reactor using 2% cellobiose, 0.1 M sodium acetate buffer, 5 pH and 50 °C. 84% cellobiose to glucose conversion was found in first cycle which was about 67% in fifth cycle. This study suggests that enzyme immobilization improved enzyme stability, substrate inhibition and permits enzyme reusability.

**Key words:** alginate, biofuel, cellulose, immobilization, kinetics

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### 1. Introduction

Bioethanol is considered as a clean fuel having vast potential to decrease our dependence on fossil fuels and reducing the greenhouse gases emissions. Globally ethanol from lignocellulosic biomass is recognised as a potential, environmentally friendly and sustainable alternative source of energy. Although, much research has been done on production of second generation bioethanol, still its commercial viability is not fully established. One of the key reasons for this is the enzymatic depolymeration of pretreated lignocellulosic biomass which involves

costly cellulolytic enzymes. Cellulases are multi-component enzyme system composed of three main groups: cellobio-hydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and  $\beta$ -glucosidases (EC 3.2.1.21). Endo and exoglucanases are known to act synergistically in the cellulose hydrolysis (Chauve et al., 2010; Stewart and Swaisgood, 1993) to produce cellobiose which is a glucose dimer and  $\beta$ -glucosidase acts on it to release glucose as an end product (Agrawal et al., 2013). Cellulase produced from *Trichoderma reesei*, generally has insufficient  $\beta$ -glucosidases activity (Dashtban and Qin, 2012). Therefore, its supplementation with exogenously

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derived  $\beta$ -glucosidases from other organisms (like *Aspergillus sp.*) is required to achieve complete biomass hydrolysis. However, supplementation may lead to additional costs. Recently developed cellulase preparations have high  $\beta$ -glucosidase and lytic polysaccharides monooxygenases (LPMO's, previously known as GH61) contents. These improvements have increased their performances manifold (Cannella and Jørgensen, 2014).

The costs of cellulase preparations from Genencor, USA and Novozymes, Denmark have been reduced significantly (20-fold) over the past decade (McMillan et al., 2011). Biomass saccharification still remains a key cost barrier and further reduction in enzyme cost is needed (Klein-Marcuschamer et al., 2012). Borges et al. (2014) reported that biocatalysts are desirable for industrial purposes because they provide greater catalytic activity per reaction volume, increasing the reaction rate and consequently decreasing the time required to achieve the conversion. High volumetric productivities are reflected in lower process costs. Thus enzyme immobilization may prove to be more useful for industrial process where, high enzyme loadings are added to hydrolyze the pretreated biomass swiftly followed by enzymes recovery and recycling.

There are four main strategies to reduce the cost of enzymatic hydrolysis of biomass. These include, a) isolation of novel high activity enzymes, b) minimizing the cost of enzyme production and c) enhancement of hydrolysis rate through synergistic effects d) reusing or recycling of enzymes for multiple rounds of hydrolysis. Over the last decade, significant research has been done on the first three strategies with few successes, but recycling of enzymes has not received much attention (Jochems et al., 2011; Pribowo et al., 2012; Reshmi and Sugunan, 2013; Srikanth et al., 2014).

Immobilization of enzyme on solid support is a good approach for enzyme recycling. As, cellulase enzyme activity has been shown to be unstable over extended period (Maheshwari et al., 2000) therefore, enzyme immobilization may improve its stability. If a simple and cheaper method for  $\beta$ -glucosidase immobilization is available then addition of exogenous  $\beta$ -glucosidase could be a possible solution to increase the rate and extent of saccharification of cellulose. Enzyme immobilization on particles could reduce the enzyme cost by improving operational stability of the enzyme and allowing re-use (Alftren and Hobley, 2013; Borges et al., 2014; Chen et al., 2014).

Various techniques have been developed for enzyme immobilization, including adsorption to insoluble materials (Shin and Rogers, 1996), entrapment in polymeric gels (Illanes et al., 1996; Shin and Rogers, 1996), encapsulation in membranes (Fang et al., 2011), crosslinking with bifunctional reagent (Husain and Jafri, 1995), or covalent linking to an insoluble carrier (Ivanova et al., 1985; Smalla et al., 1988). Of these, entrapment is one of the

simplest methods of immobilization. In recent years, the use of calcium alginate as an immobilization medium has attracted considerable attention. It has been used to immobilize a variety of cell types (Smidsrod and Skjak-Braek, 1990; Trabelsi et al., 2014), subcellular organelles (Kierstan and Bucke, 2000), enzymes (Yao et al., 2014) and multi-component systems (Kierstan and Bucke, 2000).

In the present work, a commercial  $\beta$ -glucosidase preparation has been used for studying cellobiose conversion. The  $\beta$ -glucosidase was immobilized in alginate gel beads and optimization has been carried out using response surface methodology (RSM) by central composite design of Statease Design Expert software. The effects of immobilization on the kinetic behaviour of free and immobilized  $\beta$ -glucosidase, as well as the effects of pH, temperature and storage stability are discussed. Further, batch reactor study was carried out and cellobiose conversion efficiency was determined. This study suggests that immobilization of  $\beta$ -glucosidase on alginate beads is a valuable technique for enzyme recycling and improving the enzyme stability and substrate inhibition.

## 2. Materials and methods

### 2.1. Chemicals

Solid support matrix (alginate) and liquid  $\beta$ -glucosidase (Cellobiase from *Aspergillus niger*, Novozyme 188) were procured from Sigma Aldrich, India (Cat C6105, Lot # SLBF8362V). All other chemicals and consumables were purchased from Himedia Pvt. Ltd., Mumbai, India and were of analytical grade.

### 2.2. $\beta$ -glucosidase assay

$\beta$ -glucosidase activity was assayed by the method described by Agrawal et al., (2017), Jäger et al. (2001). Total protein content was determined by Bradford method (Bradford, 1976).

### 2.3. Immobilization into Ca-alginate gel beads

Calcium alginate gel beads were prepared following the modified method of that proposed by Busto et al. (2006) by dropping a 2% sodium alginate solution (containing suitable amount of  $\beta$ -glucosidase) into a 0.2 M calcium chloride solution under continuous stirring. Sodium alginate or alginic acid, consisting of mannuronic and guluronic acid molecules ionically crosslinks by calcium between the carboxyl groups on neighbouring polygluronic acid segments (Martinsen et al., 1992) to form the beads with entrapped  $\beta$ -glucosidase. The beads were cured for 1 h in calcium chloride, washed several times with sodium acetate buffer (until no enzyme activity was observed in the final washing) and stored at 4 °C in buffer till use.

#### 2.4. Optimization of $\beta$ -glucosidase immobilization in alginate beads

The effectiveness of the immobilization of enzymes by calcium alginate is influenced by the gel properties (Stewart and Swaisgood, 1993). Optimization of immobilization conditions was done employing Response Surface Methodology (RSM) approach. A factorial, central composite design (CCD) for three factors with replicates at the centre point and star points were used in the investigation. The variables used were alginate concentration;  $\text{CaCl}_2$  concentration and  $\beta$ -glucosidase at five coded levels ( $-\infty$ , -1, 0, +1,  $+\infty$ ) were optimized based upon immobilization efficiency (Table 1). The response value (immobilization efficiency) is the average of triplicates. The statistical software package Design-Expert (Stat-Ease, Inc., Minneapolis, USA) was used for regression analysis of experimental data and to plot response surface. Analysis of variance (ANOVA) was used to estimate the statistical parameters (Kumar et al., 2014).

#### 2.5. Immobilization efficiency

The immobilization efficiency and immobilization yield were calculated as follows:

$$\text{Immobilization efficiency} = [A_f - (A_{w1} + A_{w2} + A_{w3} + \dots + A_{wi}) / A_f] \times 100$$

where  $A_f$  is the total activity of the free enzyme added and  $A_w$  is the activity in the wash solutions (1 to i) after immobilization.

#### 2.6. Estimation of cellobiose to glucose conversion using HPLC

The conversion of cellobiose to glucose was assessed by HPLC (Waters, USA) using Aminex HPX-H) column at a flow rate of 0.6 mL/min. Prior to analysis, the mobile phase, standards and samples were filtered through 0.22  $\mu\text{m}$  filters (Millipore). Absorbance was recorded at 410 nm with RI detector. Milli-Q Water (Millipore) was taken as the mobile phase.

#### 2.7. Optical imaging of the alginate bead with immobilized enzyme

The optical image of the bead was studied to visualize the shape, surface, and size of the beads using Sony (Cybershot, Japan) camera.

#### 2.8. Optimal pH and temperature

The effect of pH on  $\beta$ -glucosidase activity of both free and immobilized enzyme was studied at different pH ranging from 3.0 to 7.0 at 50°C. The buffers employed in these measurements were 0.1 M sodium acetate buffer for pH range 3.0- 5.0 and 0.1 M phosphate buffer for pH values 6.0- 7.0. For temperature optima, activity was assayed at different

temperatures ranging from 40- 70°C, at pH 5.0 using 0.1 M acetate buffer. The  $\beta$ -glucosidase activity was determined as described in Section 2.2.

#### 2.9. Effect of temperature on stability of free and immobilized $\beta$ -glucosidase

The influence of temperature on the stability of immobilized  $\beta$ -glucosidase was evaluated and related to that of the free  $\beta$ -glucosidase. Free  $\beta$ -glucosidase with buffer was considered as a control in comparison with immobilized enzyme with buffer. Both free and immobilized  $\beta$ -glucosidases were pre-incubated in acetate buffer (0.1 M, pH 5.0) at 50 °C and 60 °C for 14 days in the absence of pNPG. At regular intervals, the samples were withdrawn and  $\beta$ -glucosidase activity was determined at the optimum temperature (50 °C) as described in Section 2.2. The residual activity of the enzyme was calculated by taking the enzyme without incubation treatment (the zero hour sample) as control and its activity was considered to be 100 %. Graph was plotted between incubation time and percent of maximum activity.

#### 2.10. Kinetics study of free and immobilized $\beta$ -glucosidase

The effect of pNPG (0.1 mM-10 mM) on free and immobilized  $\beta$ -glucosidase activity was determined by measuring the reaction rates at glucose concentrations ranging from 0 to 10 mM. Lineweaver Burk plots (or double reciprocal plots) (Lineweaver and Burk, 1934) were plotted and  $K_m$  and  $V_{max}$  values for each  $\beta$ -glucosidase form i.e., free and immobilized were determined using MATLAB (version R2012a). The Lineweaver-Burk equation is given by:  $1/v = (1/V_{max}) + (K_m/V_{max} \times 1/[S])$  where,  $v$  is the rate of reaction,  $V_{max}$  represents the maximum rate achieved by the system at maximum (saturating) substrate concentrations. The Michaelis constant,  $K_m$  is the measure of the substrate concentration  $[S]$  required for effective catalysis to occur. That is, an enzyme with a high  $K_m$  requires a higher substrate concentration to achieve a given reaction velocity than an enzyme with a low  $K_m$  (Rogers and Gibon, 2014).

The inhibitor constant ( $K_i$ ) was also determined based on the Dixon Plot.  $K_i$  is the quantitative measure of potency of an inhibitor and it is the concentration  $[I]$  required to produce half of maximum inhibition. Equation by Dixon plot is given by:  $1/v = (1 + K_m/[S]) / (V_{max} \times K_i) \times [I] + 1/V_{max} (1 + K_m/[S])$ .

#### 2.11. Storage stability of free and immobilized $\beta$ -glucosidase

Free and immobilized  $\beta$ -glucosidase in acetate buffer (0.1 M, pH 5.0) were stored at 4°C and 25°C for 10 days. In this experiment, free  $\beta$ -glucosidase with buffer was taken as a control in comparison of immobilized enzyme with buffer.

**Table 1.** The variables and their levels for the central composite experimental design

Variable	Coded levels				
	-∞	-1	0	+1	+∞
Sodium alginate concentration (% w/v)	0.5	1.61	3.25	4.89	6
CaCl <sub>2</sub> concentration (M)	0.05	0.16	0.33	0.49	0.6
β-glucosidase (% v/v)	1	2.82	5.50	8.18	10

The residual β-glucosidase activity was determined at the interval of one day at 50°C and pH 5.0 as described in Section 2.2. At regular intervals, the samples were withdrawn and β-glucosidase activity was determined at the optimum temperature (50 °C) as described in Section 2.2.

The residual activity of the enzyme was calculated by taking the enzyme without incubation treatment (the zero hour sample) as control and its activity was considered to be 100 %. Graph was plotted between time and percent of maximum activity.

#### 2.12. Bench scale packed- bed enzymatic reactor for cellobiose hydrolysis

The reactor was run in batch mode for the study of the cellobiose hydrolysis. Approximately, 4,550 U of immobilized β-glucosidase beads (14.4 U/ bead) were loaded in the column reactors consisting of a glass tube (total length 43 cm, internal diameter 1.25 cm). The packed column contained a bead volume of about 86 cm<sup>3</sup> (or mL) with a void volume of 34 cm<sup>3</sup> (or mL). Conversion of cellobiose to glucose was measured using High Performance Liquid Chromatography (HPLC). The reactor was used in such a way that the product stream was mixed with the incoming substrate (2% cellobiose) stream.

The use of the batch reactor permitted a high catalytic time. The cellobiose was recycled through a peristaltic pump. The flow rate was constant (18 mL/min). The residence time was calculated to be 1.88 min.

#### 2.13. HPLC analysis for cellobiose to glucose conversion

The conversion of cellobiose to glucose was checked by HPLC (Waters Co., USA) and C18 reverse phase Aminex HPX-87P column (250 mm long x 4.4 mm i.d.; Particle size 5 μm, from BioRad, CA, USA), using Milli-Q Water (Millipore, USA) as the mobile phase. All the analyses were performed using RI detector and at a flow rate of 0.6 mL/min. The quantification was done from standard curve drawn using pure cellobiose and glucose. All solvents and distilled water (HPLC grade) were degassed before use. Prior to analysis, the mobile phase, standards and samples were filtered through 0.22 μm filters (Millipore, USA) before injecting into the HPLC column. Samples (20 μL) were injected and absorbance was recorded at 280 nm with RI-detector.

#### 2.14. Reusability of immobilized β-glucosidase and loss of enzyme due to leaching

The reusability of the immobilized preparation from batch reactor was assessed after each 24 h cycle of cellobiose hydrolysis at 50 °C under standard assay conditions. After every cycle, the immobilized β-glucosidase sample was collected from the reactor, washed with fresh acetate buffer (0.1 M, pH 5.0) and assayed for the activity as per the Section 2.2. The activity of the immobilized β-glucosidase at the start of 1<sup>st</sup> cycle was considered as 100%. Loss of the immobilized β-glucosidase due to leaching was also checked by analysing the samples collected after every 24 h batch reaction for β-glucosidase activity.

#### 2.15. Statistical analysis

Diagrams were plotted using Origin 6.0 or Excel. Each value in graphs and tables represents the mean of triplicate measurement.

### 3. Results and discussion

#### 3.1. Optimization of β-glucosidase immobilization in alginate beads

There are several parameters affecting the process of β-glucosidase immobilization in alginate beads. On the basis of initial immobilization results (data not shown here), sodium alginate concentration of 0.5% to 6% (w/v), CaCl<sub>2</sub> concentration of 0.05 to 0.6 M and concentration of β-glucosidase from 1% to 10% (v/v) were taken for optimizing the immobilization process using RSM. A total of 20 experiments were performed. The experimental design and results of 20 experiments are tabulated in Table 2.

The responses of the CCD design were fitted with a second order polynomial equation (Eq. 1). It describes the correlation between the significant variables and the response immobilization efficiency) in terms of coded value when using the model. The overall second-order polynomial equation, in terms of coded factors, can be written as follows:

$$\text{Immobilization efficiency} = + 6.61 + 0.45 * A + 0.16 * B - 0.22 * C - 0.11 * AB - 0.12 * AC + 0.39 * BC + 0.59 * A^2 + 0.52 * B^2 + 0.53 * C^2 \quad (1)$$

where, A is Sodium alginate concentration (% w/v), B is CaCl<sub>2</sub> concentration (M) and C is β-glucosidase (% v/v).

**Table 2.** Experimental set up based on central composite design and achieved immobilization efficiency

<i>Runs</i>	<i>Sodium alginate concentration (% w/v)</i>	<i>CaCl<sub>2</sub> concentration (M)</i>	<i>β-glucosidase (% v/v) or, (U/mL in reaction mixture)</i>	<i>Immobilization efficiency (%)</i>
1.00	1.81	0.14	4.42,(57.46)	62.60
2.00	4.19	0.14	4.42,(57.46)	89.80
3.00	1.81	0.41	4.42,(57.46)	62.12
4.00	4.19	0.41	4.42,(57.46)	76.64
5.00	1.81	0.14	8.58,(111.54)	50.90
6.00	4.19	0.14	8.58,(111.54)	63.30
7.00	1.81	0.41	8.58,(111.54)	70.83
8.00	4.19	0.41	8.58,(111.54)	81.60
9.00	1	0.27	6.50,(84.50)	56.23
10.00	5	0.27	6.50,(84.50)	78.42
11.00	3	0.05	6.50,(84.50)	60.74
12.00	3	0.50	6.50,(84.50)	66.05
13.00	3	0.27	3.00,(39)	71.52
14.00	3	0.27	10.00,(130)	56.78
15.00	3	0.27	6.50,(84.50)	45.23
16.00	3	0.27	6.50,(84.50)	42.82
17.00	3	0.27	6.50,(84.50)	48.90
18.00	3	0.27	6.50,(84.50)	38.56
19.00	3	0.27	6.50,(84.50)	43.56
20.00	3	0.27	6.50,(84.50)	43.86

The Prob>F value for the model was <0.0001, which indicated that the model was statistically significant. The Model F-value of 32.30 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. An ANOVA was performed for the evaluation of the effects of the variables and their possible interactions (Table 3). Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, BC, A<sup>2</sup>, B<sup>2</sup>, C<sup>2</sup> are significant model terms. The "Predicted R<sup>2</sup>" of 0.8657 is in reasonable agreement with the "adjusted R<sup>2</sup>" of 0.9368. This indicated a good agreement between the experimental and predicted values (Pandiyani et al., 2014; Wattanachaisaereekul et al., 2014).

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 16.370 indicates an adequate signal. Therefore, this model can be used to navigate the design space. The 3D response surface plots were obtained by plotting the response (immobilization efficiency) on the Z-axis against any other two variables. Response surface plots were prepared for better understanding of the interactions among the three variables i.e. calcium chloride, sodium alginate β- glucosidase (Fig. 1). The immobilization efficiency was found to be 89.8% for optimized conditions i.e., sodium alginate 4.19% (w/v), calcium chloride 0.14 M and β- glucosidase 4.42% (v/v) or 57.46 U/mL of reaction

mixture. High immobilization efficiency signifies that a proper association occurred between the enzyme and the support without affecting the enzyme integrity. Stewart and Swaisgood (1993) have studied the effects of different concentrations of alginate and CaCl<sub>2</sub>, on the bead formation and they have found that maximum enzyme activity (66%) was found, when 3% sodium alginate and 0.2 M calcium chloride were used for immobilization. A similar high level of activity was obtained by Kamboj et al. (1996) who immobilized cathepsin B in calcium alginate, using an alginate concentration of 3% or above.

Although, they found that the same amount of active enzyme was entrapped when using 3.3, 3.5 and 4.0% (w/v) alginate, the beads formed in those cases did not have a uniform size because of the high viscosity of the enzyme-alginate mixture. Ortega et al. (1998) have suggested that the structure and molecular weight of alginates differ with different kinds, ages and parts of seaweeds used and with different alginate extraction processes which could affect the immobilization efficiency.

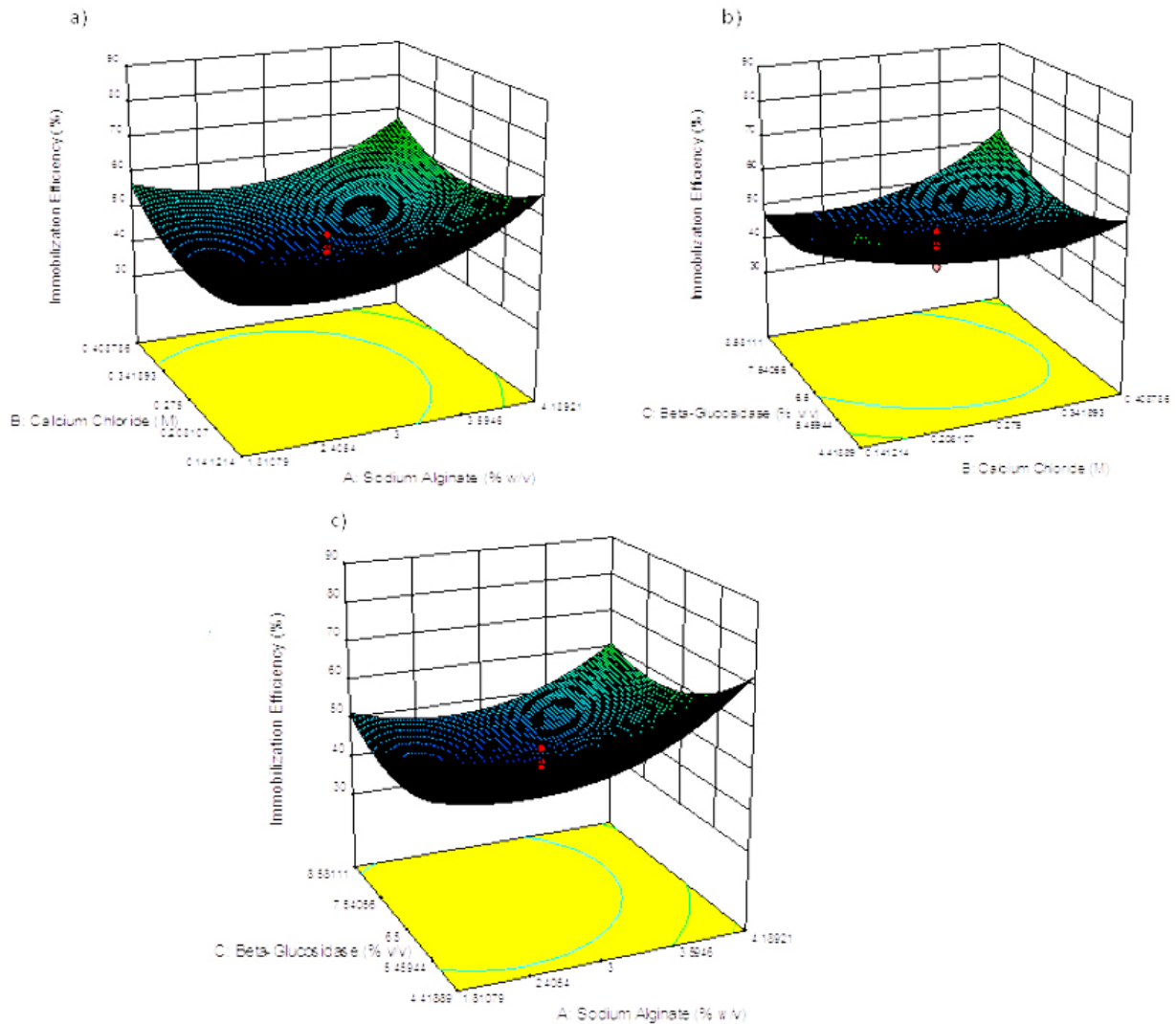
### 3.2. Optical imaging of immobilized β-glucosidase in beads

Spherical and regular-shaped alginate beads were obtained after immobilization. The diameters of the beads were 2.5 - 3.0 mm (Fig. 2).

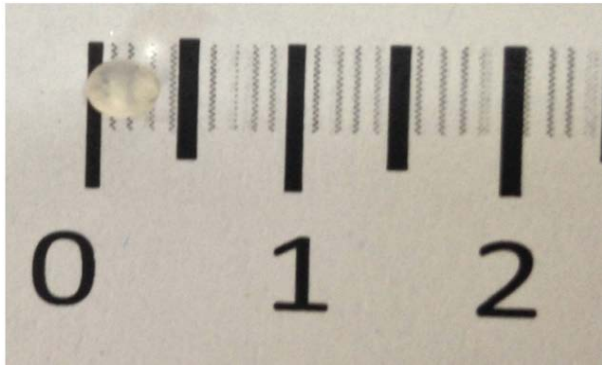
**Table 3.** ANOVA for response surface quadratic model

Source	Sum of Squares	Degree of freedom	F value	P-value (Prob>F)
Model	16.00	9	32.30	< 0.0001
A-Sodium Alginate	2.78	1	50.43	< 0.0001
B-Calcium Chloride	0.34	1	6.25	0.0315
C-Beta-Glucosidase	0.67	1	12.26	0.0057
AB	0.10	1	1.82	0.2067
AC	0.12	1	2.26	0.1633
BC	1.21	1	21.97	0.0009
A <sup>2</sup>	5.05	1	91.74	< 0.0001
B <sup>2</sup>	3.83	1	69.54	< 0.0001
C <sup>2</sup>	4.02	1	72.99	< 0.0001
Residual	0.55	10		
Lack of Fit	0.23	5	0.70	0.6498
Pure Error	0.32	5	0.065	
Cor Total	16.55	19		

R<sup>2</sup> = 0.9667; adjusted R<sup>2</sup> = 0.9368



**Fig. 1.** Response surface plots of the central composite design for the optimization of the immobilization efficiency. a) Effect of concentrations of calcium chloride and sodium alginate, b) Effect of concentrations of  $\beta$ -glucosidase and calcium chloride and c) Effect of concentrations of  $\beta$ -glucosidase and sodium alginate



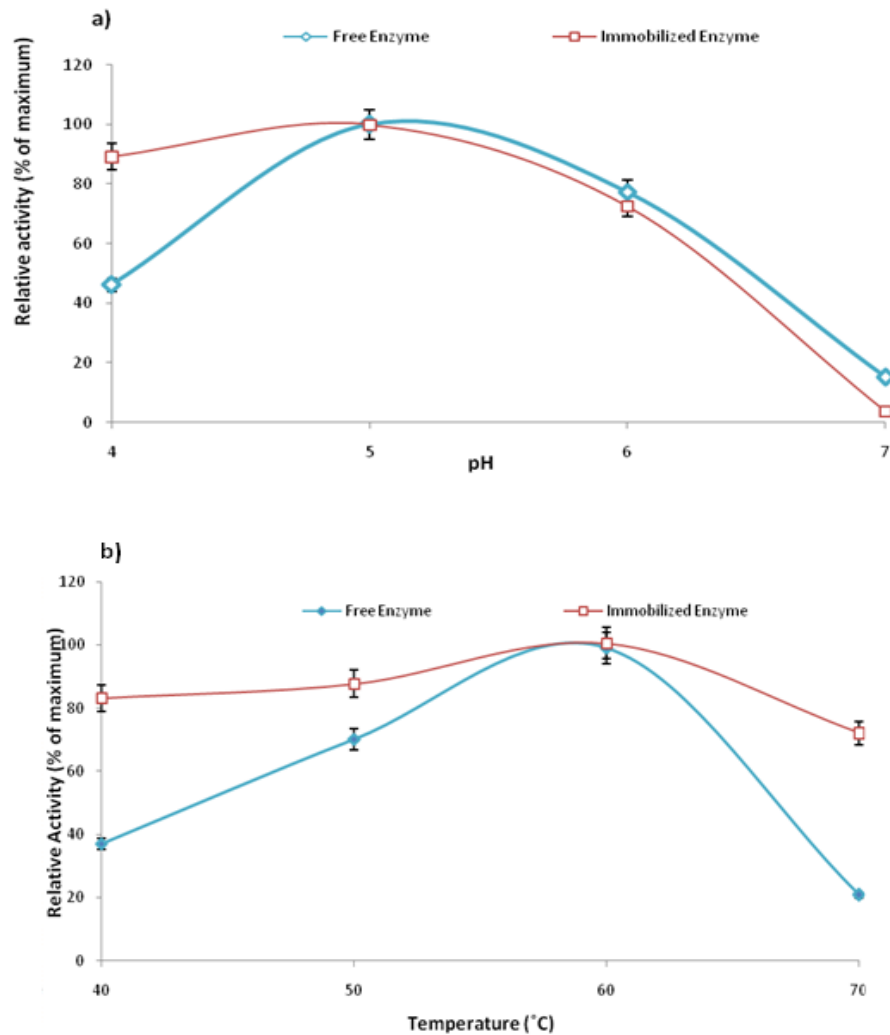
**Fig. 2.** Bead size in millimeter scale

### 3.3. Optimal pH and temperature

The effect of pH on  $\beta$ -glucosidase activity of native and immobilized enzyme was studied using different buffer solutions with pH values ranging from 3.0 to 7.0. The optimum pH for both the free and immobilized  $\beta$ -glucosidase was found to be 5.0 (Fig. 3a). Both the  $\beta$ -glucosidases were shown to be sensitive to pH higher than 5.0 and lost almost

full activity at pH 7.0. At pH 4.0 though free  $\beta$ -glucosidase was inactive but immobilized  $\beta$ -glucosidase showed remarkable activity. Previous studies have also reported pH around 5.0 to be optimum for the  $\beta$ -glucosidases isolated from different bacterial and plant systems (Han and Chen, 2008; Hsieh and Graham, 2001; Sue et al., 2000). The activity of these  $\beta$ -glucosidases also decreased rapidly below pH 4.5 and above pH 5.0. Total loss of the activity was observed at pH 6.0. Previous reports (Bhatia et al., 2002; Riou et al., 1998) have also reported the pH optima from 5.0 to 6.5 for  $\beta$ -glucosidase isolated from fungus. An acidic pH optima and maximal activity at about pH 4.5 has been reported as common features of  $\beta$ -glucosidase enzymes isolated from diverse microbial systems (Leite et al., 2007).

Both free and immobilized  $\beta$ -glucosidases showed maximal activity at 60 °C (Fig. 3b). Singh et al. (2011) also observed that the optimum temperature of the free  $\beta$ -glucosidase, isolated from *Agaricus arvensis* was 65 °C, but the  $\beta$ -glucosidase immobilized on SiO<sub>2</sub> nanoparticles had maximal activity at 70 °C.



**Fig. 3.** a) pH optima of free and immobilized  $\beta$ -glucosidase at 50 °C, b) Temperature optima of free and immobilized  $\beta$ -glucosidase at pH 5.0.

It was also observed that for the free enzyme, increase in temperature resulted in a sharp decrease in the relative activity of the free enzyme. In contrast, the immobilized enzyme showed no such tendency and retained 82% of its optimum activity even at 85 °C. Maheshwari et al. (2000) also observed that the most of the thermostable  $\beta$ -glucosidases obtained from different sources have an optimum temperature between 45 °C and 80 °C. While,  $\beta$ -glucosidases isolated from corn stover showed maximal activity around 37 °C and showed low activity below 30 °C and above 40 °C (Han and Chen, 2008).

### 3.4. Stability of free and immobilized $\beta$ -glucosidase

Enzyme stability at high temperatures is usually desirable for industrial applications but enzymes are prone to lose their activity at high temperatures because proteins suffer structural changes leading to protein aggregation and precipitation (Rosales-Calderon et al., 2014). Thus thermostability of both free and immobilized  $\beta$ -glucosidases was determined at two different temperatures (50 °C and 60 °C) at pH 5.0. At 50 °C, free enzyme has lost approx. 20% of its activity after 2h. Thereafter, it remains constant till 24 h and a 12% decline in its activity was observed from 24 h to 4<sup>th</sup> day (96 h). Further, approx 20% decrease in its activity was observed from 4<sup>th</sup> day to 10<sup>th</sup> day. Ultimately, 83% loss from its maximum (at 0 h) its activity was observed after 14 days. Immobilized enzyme has lost approx. 10% of its activity in initial 2 h after this it remained almost constant till 2<sup>nd</sup> day. Thereafter, it declined after 4<sup>th</sup> day (52%) and finally it reduced to 60% after 14 days (Fig. 4).

However, at 60°C the free  $\beta$ -glucosidase enzyme has lost almost 25% of its activity after 2 h thereafter, it gradually declined to 33% at 24 h. Free enzyme has lost its complete activity at 3<sup>rd</sup> day. But immobilized enzyme has gradually lost about 24% activity between 1h to 24 h. Then it suddenly dropped to 70% of its maximum (at 0 h) activity after 2<sup>nd</sup> day and loosing approx. 87% of its activity after 14 days. Results suggest that both free and immobilized enzymes gradually lose their activity with time but enzymes were more stable at 50°C than 60°C. Moreover, immobilized enzyme was found to be more stable than free enzyme at both temperatures (50 °C and 60°C). This increase in stability after immobilization may be attributed to the multiple covalent bonds that alter the conformational flexibility and thermal vibrations, thus preventing the immobilized protein from unfolding and denaturing (Wong et al., 2009). Tu et al. (2006) has reported that free  $\beta$ -glucosidase (Novozyme 188) has lost almost 90% of its activity after 10 h at 65 °C. While immobilized  $\beta$ -glucosidase was more stable retaining almost 55% activity under same conditions.

Dekker (1986) reported that Novozyme 188 lost 17 and 72% of its  $\beta$ -glucosidase activity after 48 and 120 h incubation at 50 °C. Rosales-Calderon et al.

(2014) reported that protein concentration of Novozyme 188 decreased by 77% in 4 days at 50°C. Christakopoulos et al. (1994); Dekker (1986); Karnchanatat et al. (2007); Yan and Lin (1997); Yazdi et al. (2003) have also reported that temperature stability of  $\beta$ -glucosidases ranges between 50 to 65 °C. The  $\beta$ -glucosidase from fungus *D. eschscholzii* was also found to be stable for only 30 min at 60 °C (Karnchanatat et al., 2007). Singh et al. (2011) reported about 288-fold increase in the thermal stability at 65 °C, after immobilization of  $\beta$ -glucosidase on SiO<sub>2</sub> nanoparticles.

### 3.5. Kinetics study of free and immobilized $\beta$ -glucosidase

Kinetic parameters of free and immobilized  $\beta$ -glucosidases were determined by measuring the initial reaction rates with pNPG (0.1 mM-10 mM) as substrate and varying amounts of glucose as inhibitor (0 mM- 10 mM). The Michaelis constants ( $K_m$ ) and  $V_{max}$  values for the free and insoluble  $\beta$ -glucosidases were obtained by MATLAB (version R2012a).  $K_m$  is equal to the substrate concentration at which the rate is half of the maximum rate. It represents the substrate concentration at which half the enzyme active sites are filled by substrate molecules. While  $V_{max}$  represents the maximum rate attainable; that is, it is the rate at which the total enzyme concentration is present as the enzyme- substrate complex. As shown in Fig. 5, a series of lines crosses the y (1/v) axis at the same point (i.e. an unchanged and constant  $V_{max}$ ) with a decreasing value of 1/ $K_m$  (or increasing  $K_m$ ) in the presence of the inhibitor. This indicates competitive mode of inhibition by glucose for both the free and immobilized  $\beta$ -glucosidases. As shown in Fig. 5, a series of lines crosses the y (1/v) axis at the same point (i.e. an unchanged and constant  $V_{max}$ ) with a decreasing value of 1/ $K_m$  (or increasing  $K_m$ ) in the presence of the inhibitor. This indicates competitive mode of inhibition by glucose for both the free and immobilized  $\beta$ -glucosidases.

$K_m$  value of the immobilized  $\beta$ -glucosidase (0.995 mM) was found to be slightly more than that of the free  $\beta$ -glucosidase (0.617 mM).  $K_m$  value of free *Novozymes 188*  $\beta$ -glucosidases has been reported as 1.03 mM when using pNPG as substrate (Chauve et al., 2010). Higher values of  $K_m$  (10-fold higher) for the immobilized  $\beta$ -glucosidase versus free enzyme have also been reported earlier (Tu et al., 2006). Georgelin et al. (2010) observed that  $K_m$  increased (from 7.6 mM to 8.6 mM) after immobilization of  $\beta$ -glucosidase on functionalized  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> @ SiO<sub>2</sub> core-shell magnetic nanoparticles. Increased Michaelis constant,  $K_m$  indicates that immobilization has slightly altered the affinity of the  $\beta$ -glucosidase for its substrate. The enzyme entrapment in the alginate is supposed to alter the diffusion rate and accessibility of the substrate to the active site of enzyme (Singh et al., 2011).



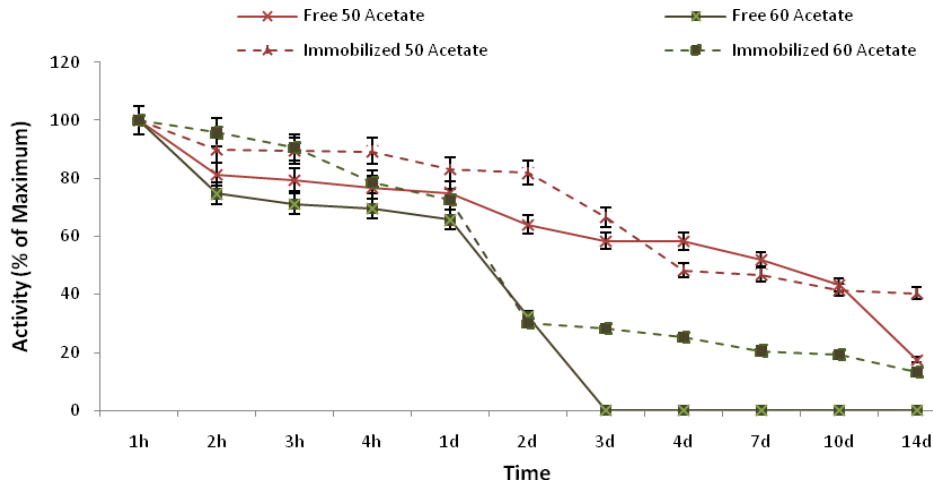
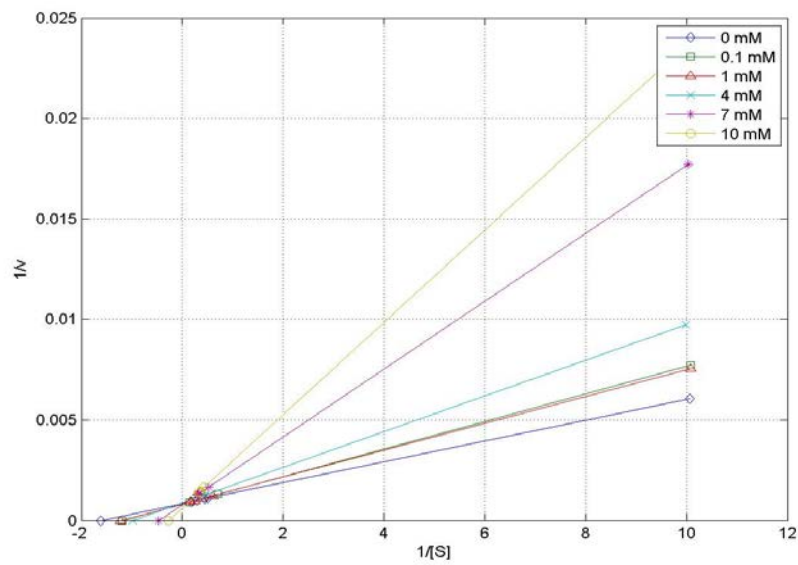
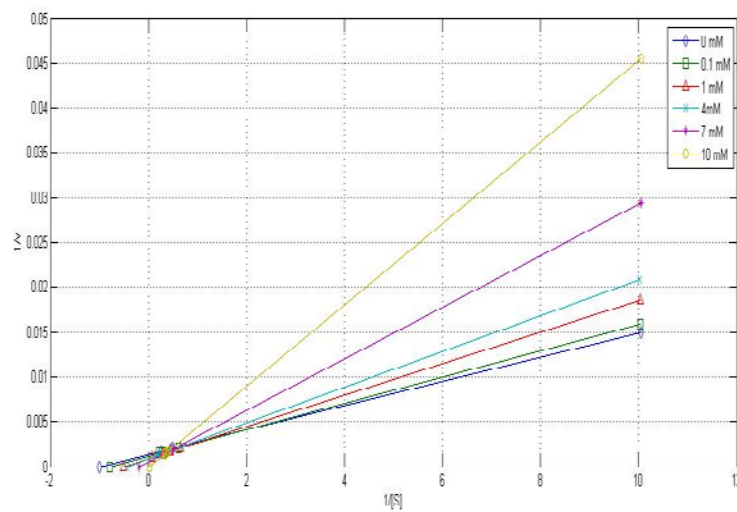


Fig. 4. Stability of free and immobilized  $\beta$ -glucosidase at 50 °C and 60 °C using acetate buffer at pH 5.0



a)



b)

Fig. 5. Mode of  $\beta$ -glucosidase inhibition by glucose. Lineweaver-Burk plot of  $\beta$ -glucosidase inhibition by glucose. Free  $\beta$ -glucosidase was treated with various concentrations of pNPG (0.1-10 mM) in the absence or presence of glucose (0-10 mM). The kinetics assay has been performed after incubating the mixture at 50 °C for 30 min. a) free  $\beta$ -glucosidase b) immobilized  $\beta$ -glucosidase

On the other hand, the  $V_{max}$  of the immobilized  $\beta$ -glucosidases was smaller ( $736.65 \mu\text{mol min}^{-1}$ ) than that of the native  $\beta$ -glucosidase ( $1191.74 \mu\text{mol min}^{-1}$ ). In a recent report, immobilization of  $\beta$ -glucosidase on  $\text{SiO}_2$  nanoparticles caused an increase in both the  $K_m$  (2.5 mM for free and 3.8 mM for immobilized) and  $V_{max}$  (3028 U/mg protein to 3347 U/mg protein) of the enzyme. This indicates increased mass-transfer limitation for the substrate after immobilization due to the reduced flexibility and deformation of  $\beta$ -glucosidase enzyme molecules after being entrapped (Singh et al., 2011).

It is always desirable for an enzyme to have low  $K_m$  value (enzyme with a low  $K_m$  would require a lower substrate concentration to achieve half of the maximum reaction velocity than an enzyme with a high  $K_m$ ) and high  $V_{max}$  (maximum reaction rate). Immobilization creates a hindrance or resistance which affects enzyme affinity for its substrate. As a result of which,  $K_m$  usually increases as compared to its free counterpart, while  $V_{max}$  may either decrease or increase or may remain unchanged. But immobilization also imparts certain benefits like stability and recyclability. To confirm the mode of inhibition of enzyme by glucose, the Dixon plots were plotted for both the free and immobilized  $\beta$ -glucosidases (Fig. 6). Plotting  $1/v$  against concentration of inhibitor at each concentration of substrate (the Dixon plot) gives a family of intersecting lines. For a competitive inhibitor, the lines converge above the x axis, and the value of inhibitor concentration where they intersect is  $-K_i$ . The inhibitor constant,  $K_i$ , is an indication of how potent an inhibitor is; it is the concentration required to produce half of the maximum inhibition.

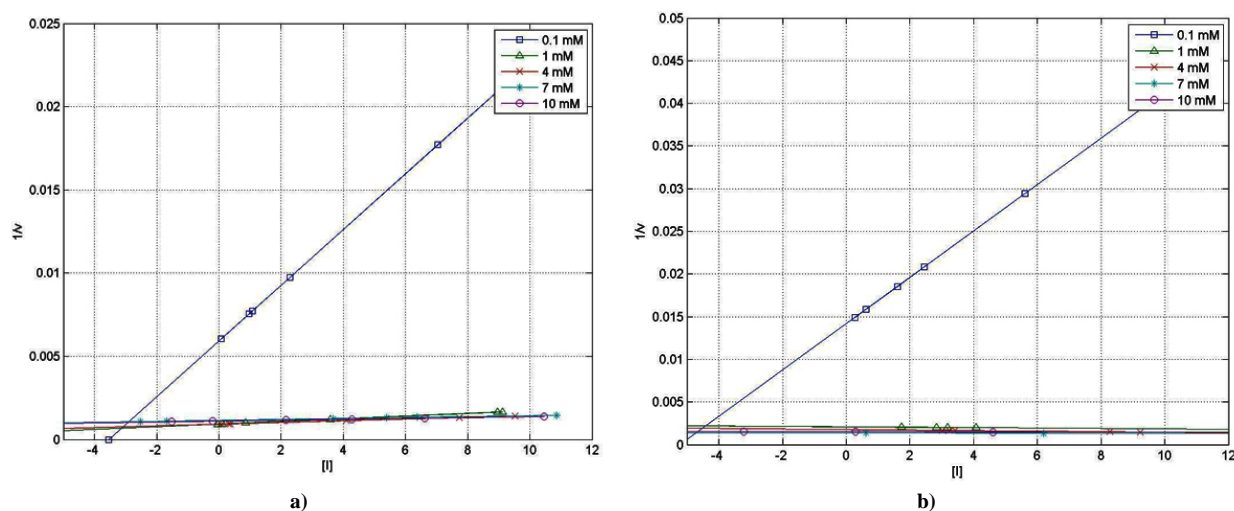
The curve generated for free  $\beta$ -glucosidases showed a competitive mode of  $\beta$ -glucosidase

inhibition by glucose ( $K_i = 3.01 \text{ mM}$ ). This implies that at low substrate concentration, the glucose molecule compete with the substrate for binding to the active site of the enzyme forming an inactive complex with enzyme, thereby preventing or slowing down the breakdown of glycosides to glucose.

This is in agreement with previous reports that glucose is a strong inhibitor of  $\beta$ -glucosidase (Chauve et al., 2010). After immobilization the inhibition was of competitive type but reduced ( $K_i = 4.56 \text{ mM}$ ) indicating that the diffusion effect increased not only the  $K_m$ , but also the threshold for substrate inhibition.  $K_i$  value of free *Novozymes 188*  $\beta$ -glucosidases has been reported as 3 mM when using pNPG as substrate and glucose as inhibitor (Chauve et al., 2010). He also found the similar results showing competitive inhibition of  $\beta$ -glucosidase by glucose.

### 3.6. Storage stability of free and immobilized $\beta$ -glucosidase

Free and immobilized  $\beta$ -glucosidases were stored in acetate buffer (0.1 M, pH 5.0) at  $4^\circ\text{C}$  and  $25^\circ\text{C}$  for 15 days. The residual  $\beta$ -glucosidase activity was determined at  $50^\circ\text{C}$  and pH 5.0 as described in Section 2.2. The storage stability of immobilized  $\beta$ -glucosidase increased as compared to the free  $\beta$ -glucosidase (Fig. 7). Immobilized  $\beta$ -glucosidase retained about 70% residual activity when stored at  $4^\circ\text{C}$  and 64% residual activity at  $25^\circ\text{C}$  after 15 days. While the free  $\beta$ -glucosidase had only 35% residual activity when stored at  $4^\circ\text{C}$  and 10% residual activity at  $25^\circ\text{C}$  after 15 days. Zhou et al. (2013) reported that free  $\beta$ -glucosidase (Novozyme 188) in  $\text{Na}_2\text{HPO}_4\text{-C}_6\text{H}_8\text{O}_7$  buffer lost about 80% of its activity in 6 weeks while,  $\beta$ -glucosidase immobilized on magnetic  $\text{Fe}_3\text{O}_4$  nanoparticles lost only 20% of its activity in same time at  $4^\circ\text{C}$ .



**Fig. 6.** Dixon plot of  $\beta$ -glucosidase inhibition by glucose. Each line represents linear regression analysis of reciprocal of average rates of product formation as a function of inhibitor concentration for different substrate concentrations: 0 mM, 0.1 mM, 1 mM, 4 mM, 7 mM, and 10 mM. The kinetics assay has been performed after incubating the mixture at  $50^\circ\text{C}$  for 30 min. a) free  $\beta$ -glucosidase b) immobilized  $\beta$ -glucosidase

Similarly, R and S (2013) reported that free  $\beta$ -glucosidase (Novozyme 188) in acetate buffer lost about 85% of its activity in 10 days while,  $\beta$ -glucosidase immobilized on nannoporous silica foam retained almost full activity even after 60 days at 4 °C. At 25 °C free enzyme lost 100% activity in 6 days while immobilized enzyme were more stable for long period.

### 3.7. Cellobiose hydrolysis using packed bed reactor in batch mode

Upto 85% conversion of cellobiose to glucose in first batch and 67% conversion in fifth batch after 24 h was achieved (Fig. 8). There was approximately 8.5% decrease in glucose conversion in second batch and it further reduced to 12.7% after third batch and finally to 17.8% decrease after fifth batch. Tu et al. (2006) have also reported that 80% hydrolysis was achieved in first batch which was reduced to 75% in second batch and remained

relatively stable during the subsequent four rounds.

### 3.8. Reusability of free and immobilized $\beta$ -glucosidase

The reusability of an immobilized enzyme preparation depends on certain factors i.e., the operation time, operation temperature and the process. Reusability of immobilized  $\beta$ -glucosidase was evaluated for 5 consecutive batch reactions each of 24 h. After each batch, the immobilized enzyme sample was collected from the reactor, washed with fresh acetate buffer (0.1 M, pH 5.0) and assessed for the remaining activity.

The activity of the immobilized enzyme in the 0th cycle was considered as 100%. The immobilized  $\beta$ -glucosidase retained almost 82% of its original activity after 5<sup>th</sup> batch i.e. 120 h (Fig. 9). This is in agreement with the previous results showing cellobiose hydrolysis using batch mode reactor as loss in hydrolysis (17.7%) matches the loss in enzyme activity (17.8%).

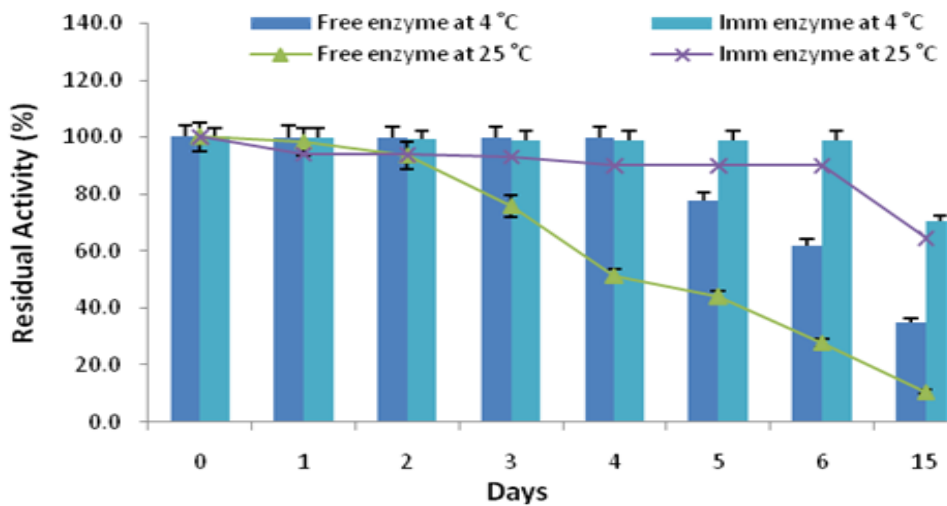


Fig. 7. Storage stability of free and immobilized  $\beta$ -glucosidase at 4 °C and 25 °C

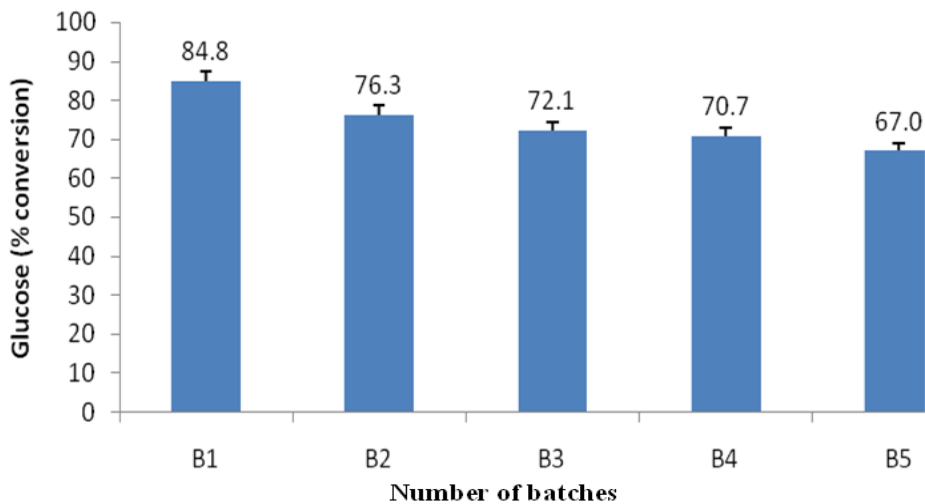


Fig. 8. Cellobiose hydrolysis using batch mode reactor for a period of 24 h

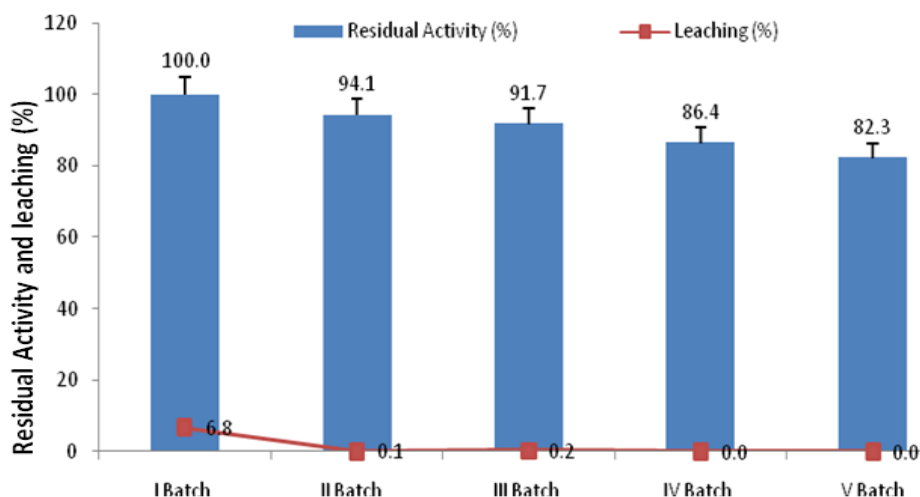


Fig. 9. Reusability of immobilized enzyme and leaching during batch reactions

Su et al. (2010) used the  $\beta$ -glucosidase immobilized on sodium alginate repeatedly for about 100 h with 93.6% of residual activity at 45 °C. The reusability of  $\beta$ -glucosidase immobilized on silica gel exhibited a half-life of about 130 h at a flow rate of 1.1–1.2 mL/min at 75 °C. Singh et al. (2011) immobilized *Agaricus arvensis*  $\beta$ -glucosidase on SiO<sub>2</sub> nanoparticles which exhibited 95% of its original activity after 25th cycle of reuse but no hydrolysis experiments were conducted prior to activity analyses. Also, the loss of the enzyme due to leaching was found to be only about 7% during whole process. This suggested that the leaching was not the cause for the reduced enzyme activity.

#### 4. Conclusions

High immobilization efficiency (about 89.6%) was achieved under optimized immobilization conditions. Immobilization also improved the thermal stability and reduced the substrate inhibition of enzyme. About 84% cellobiose to glucose conversion in first cycle and 67% conversion after fifth cycle was achieved using bench- scale batch reactor. Thus, after immobilization,  $\beta$ -glucosidase could be used for multiple cycles of hydrolysis without any significant loss in its activity.

Enzyme immobilization provides an alternative solution for an industrial process where, high enzyme loadings could be used for hydrolyzing the biomass swiftly followed by enzymes recycling. Thus it is highly process dependent to use fresh enzymes every time if it is economical or to use the immobilized enzyme.

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#### References

- Agrawal R., Satlewal A., Verma A., (2013a), Utilization of citrus sinensis waste for the production of  $\beta$ -glucosidase by solid-state fermentation using a bacillus subtilis mutant, *Environmental Engineering and Management Journal*, **16**, 1465-1471.
- Agrawal R., Satlewal A., Verma A., (2013b), Development of a  $\beta$ -glucosidase hyperproducing mutant by combined chemical and UV mutagenesis, *3 Biotech*, **3**, 381-388.
- Alftren J., Hobley T.J., (2013), Covalent immobilization of beta-glucosidase on magnetic particles for lignocellulose hydrolysis, *Applied Biochemistry and Biotechnology*, **169**, 2076-87.
- Bhatia Y., Mishra S., Bisaria V.S., (2002), Microbial beta-glucosidases: cloning, properties, and applications, *Critical Reviews in Biotechnology*, **22**, 375-407.
- Borges D.G., Baraldo Junior A., Farinas C.S., de Lima C. G.R., Tardioli P.W., (2014), Enhanced saccharification of sugarcane bagasse using soluble cellulase supplemented with immobilized beta-glucosidase, *Bioresource Technology*, **167**, 206-13.
- Bradford M.M., (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry*, **72**, 248-254.
- Busto M.D., Garcia-Tramontin K.E., Ortega N., Perez-Mateos M., (2006), Preparation and properties of an immobilized pectinase for the treatment of fruit juices, *Bioresource Technology*, **97**, 1477-1483.
- Cannella D., Jørgensen H., (2014), Do new cellulolytic enzyme preparations affect the industrial strategies for high solids lignocellulosic ethanol production?, *Biotechnology and Bioengineering*, **111**, 59-68.
- Chauve M., Mathis H., Huc D., Casanave D., Monot F., Lopes Ferreira N., (2010), Comparative kinetic analysis of two fungal beta-glucosidases, *Biotechnology for Biofuels*, **3**, 3.
- Chen T., Yang W., Guo Y., Yuan R., Xu L., Yan Y., (2014), Enhancing catalytic performance of beta-glucosidase via immobilization on metal ions chelated magnetic nanoparticles, *Enzyme and Microbial Technology*, **63**, 50-57.
- Christakopoulos P., Goodenough P.W., Kekos D., Macris B. J., Claeysens M., Bhat M.K., (1994), Purification and characterisation of an extracellular beta-glucosidase with transglycosylation and exo-glucosidase activities

- from *Fusarium oxysporum*, *European Journal of Biochemistry*, **224**, 379-385.
- Dashtban M., Qin W., (2012), Overexpression of an exotic thermotolerant beta-glucosidase in *trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw, *Microbial Cell Factories*, **11**, 63, doi: 10.1186/1475-2859-11-63.
- Dekker R.F., (1986), Kinetic, inhibition, and stability properties of a commercial beta-D-glucosidase (cellobiase) preparation from *aspergillus niger* and its suitability in the hydrolysis of lignocellulose, *Biotechnology and Bioengineering*, **28**, 1438-1442.
- Fang Y., Huang X.J., Chen P.C., Xu Z.K., (2011), Polymer materials for enzyme immobilization and their application in bioreactors, *BMB reports*, **44**, 87-95.
- Georgelin T., Maurice V., Malezieux B., Siaugue J.-M., Cabuil V., (2010), Design of multifunctionalized  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub> core-shell nanoparticles for enzymes immobilization, *Journal of Nanoparticle Research*, **12**, 675-680.
- Han Y., Chen H., (2008), Characterization of beta-glucosidase from corn stover and its application in simultaneous saccharification and fermentation, *Bioresource Technology*, **99**, 6081-6087.
- Hsieh M.C., Graham T.L., (2001), Partial purification and characterization of a soybean beta-glucosidase with high specific activity towards isoflavone conjugates, *Phytochemistry*, **58**, 995-1005.
- Husain S., Jafri F., (1995), Covalent immobilization of invertase and horseradish peroxidase on concanavalin A-Serulose via carbohydrate moieties, *Biochemistry and Molecular Biology International*, **36**, 669-677.
- Illanes A., Altamirano C., Zuniga M.E., (1996), Thermal inactivation of immobilized penicillin acylase in the presence of substrate and products, *Biotechnology and Bioengineering*, **50**, 609-616.
- Ivanova L.A., Rakhimov M.M., El'chits S.V., Ruzieva D.M., Sandul G.V., (1985), Activity and stability of glucoamylase preparations in different methods of immobilization, *Ukrainskii Biokhimičeskii Zhurnal*, **57**, 31-36.
- Jäger S., Brumbauer A., Fehér E., Réczey K., Kiss L., (2001), Production and characterization of  $\beta$ -glucosidases from different *Aspergillus* strains, *World Journal of Microbiology and Biotechnology*, **17**, 455-461.
- Jochems P., Satyawali Y., Diels L., Dejonghe W., (2011), Enzyme immobilization on/in polymeric membranes: status, challenges and perspectives in biocatalytic membrane reactors (BMRs), *Green Chemistry*, **13**, 1609-1623.
- Kamboj R.C., Raghav N., Nandal A., Singh H., (1996), Properties of cathepsin B immobilized in calcium alginate beads, *Journal of Chemical Technology and Biotechnology*, **65**, 149-155.
- Karnchanat A., Petsom A., Sangvanich P., Piaphukiew J., Whalley A.J., Reynolds C. D., Sihanonth P., (2007), Purification and biochemical characterization of an extracellular beta-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm, *FEMS Microbiology Letters*, **270**, 162-170.
- Kierstan M., Bucke C., (2000), The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels, *Biotechnology and Bioengineering*, **19**, 387-397.
- Klein-Marcuschamer D., Oleskowicz-Popiel P., Simmons B.A., Blanch H.W., (2012), The challenge of enzyme cost in the production of lignocellulosic biofuels, *Biotechnology and Bioengineering*, **109**, 1083-1087.
- Kumar R., Satlewal A., Sharma S., Kagdiyal V., Gupta R. P., Tuli D. K., Malhotra R. K., (2014), Investigating *Jatropha* prunings as a feedstock for producing fermentable sugars and chemical treatment for process optimization, *Journal of Renewable and Sustainable Energy*, **6**, 0331181, <https://doi.org/10.1063/1.4880215>.
- Leite R. S., Bocchini D. A., Martins Eda S., Silva D., Gomes E., Da Silva R., (2007), Production of cellulolytic and hemicellulolytic enzymes from *Aureobasidium pullans* on solid state fermentation, *Applied Biochemistry and Biotechnology*, **137-140**, 281-288.
- Lineweaver H., Burk D., (1934), The determination of enzyme dissociation constants, *Journal of the American Chemical Society*, **56**, 658-666.
- Maheshwari R., Bharadwaj G., Bhat M.K., (2000), Thermophilic fungi: their physiology and enzymes, *Microbiology and Molecular Biology Reviews*, **64**, 461-488.
- Martinsen A., Storro I., Skjark-Braek G., (1992), Alginate as immobilization material: III. Diffusional properties, *Biotechnology and Bioengineering*, **39**, 186-94.
- McMillan J.D., Jennings E.W., Mohagheghi A., Zuccarello M., (2011), Comparative performance of precommercial cellulases hydrolyzing pretreated corn stover, *Biotechnology for Biofuels*, **4**, 29, <https://doi.org/10.1186/1754-6834-4-29>.
- Ortega N., Busto M. D., Perez-Mateos M., (1998), Optimisation of  $\beta$ -glucosidase entrapment in alginate and polyacrylamide gels, *Bioresource Technology*, **64**, 105-111.
- Pandiyan K., Tiwari R., Singh S., Nain P.K., Rana S., Arora A., Singh S.B., Nain L., (2014), Optimization of enzymatic saccharification of alkali pretreated *Parthenium sp.* using Response Surface Methodology, *Enzyme Research*, ID 764898, <http://dx.doi.org/10.1155/2014/764898>.
- Pribowo A., Arantes V., Saddler J.N., (2012), The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulase/xylanase components when hydrolyzing steam pretreated corn stover, *Enzyme and Microbial Technology*, **50**, 195-203.
- Reshmi R., Sugunan S., (2013), Improved biochemical characteristics of crosslinked  $\beta$ -glucosidase on nanoporous silica foams, *Journal of Molecular Catalysis B: Enzymatic*, **85-86**, 111-118.
- Riou C., Salmon J. M., Vallier M. J., Gunata Z., Barre P., (1998), Purification, characterization, and substrate specificity of a novel highly glucose-tolerant beta-glucosidase from *Aspergillus oryzae*, *Applied and Environmental Microbiology*, **64**, 3607-3614.
- Rogers A., Gibon Y., (2014), *Enzyme Kinetics: Theory and Practice*, In: *Plant Metabolic Networks*, Schwender J. (Ed.), Springer Science+Business Media, 71-103.
- Rosales-Calderon O., Trajano H. L., Duff S. J., (2014), Stability of commercial glucanase and beta-glucosidase preparations under hydrolysis conditions, *PeerJ*, **2**, e402, doi: 10.7717/peerj.402. eCollection 2014.
- Shin H.S., Rogers P.L., (1996), Kinetic evaluation of biotransformation of benzaldehyde to L-phenylacetylcarbinol by immobilized pyruvate decarboxylase from *Candida utilis*, *Biotechnology and Bioengineering*, **49**, 429-436.
- Singh R.K., Zhang Y.W., Nguyen N.P., Jeya M., Lee J.K., (2011), Covalent immobilization of beta-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles, *Applied Microbiology and Biotechnology*, **89**, 337-344.

- Smalla K., Turkova J., Coupek J., Hermann P., (1988), Influence of salts on the covalent immobilization of proteins to modified copolymers of 2-hydroxyethyl methacrylate with ethylene dimethacrylate, *Biotechnology and Applied Biochemistry*, **10**, 21-31.
- Smidsrod O., Skjak-Braek G., (1990), Alginate as immobilization matrix for cells, *Trends in Biotechnology*, **8**, 71-78.
- Srikanth S., Maesen M., Dominguez-Benetton X., Vanbroekhoven K., Pant D., (2014), Enzymatic electrosynthesis of formate through CO<sub>2</sub> sequestration/reduction in a bioelectrochemical system (BES), *Bioresource Technology*, **165**, 350-354.
- Stewart W.W., Swaisgood H.E., (1993), Characterization of calcium alginate pore diameter by size-exclusion chromatography using protein standards, *Enzyme and Microbial Technology*, **15**, 922-927.
- Su E., Xia T., Gao L., Dai Q., Zhang Z., (2010), Immobilization of  $\beta$ -glucosidase and its aroma-increasing effect on tea beverage, *Food and Bioproducts Processing*, **88**, 83-89.
- Sue M., Ishihara A., Iwamura H., (2000), Purification and characterization of a beta-glucosidase from rye (*Secale cereale* L.) seedlings, *Plant Science*, **155**, 67-74.
- Trabelsi I., Ayadi D., Bejar W., Bejar S., Chouayekh H., Ben Salah R., (2014), Effects of *Lactobacillus plantarum* immobilization in alginate coated with chitosan and gelatin on antibacterial activity, *International Journal of Biological Macromolecules*, **64**, 84-89.
- Tu M., Zhang X., Kurabi A., Gilkes N., Mabee W., Saddler J., (2006), Immobilization of beta-glucosidase on Eupergit C for lignocellulose hydrolysis, *Biotechnology Letters*, **28**, 151-156.
- Wattanachaisaereekul S., Tachaleat A., Punya J., Haritakun R., Boonlarppradab C., Cheevadhanarak S., (2014), Assessing medium constituents for optimal heterologous production of anhydromevalonolactone in recombinant *Aspergillus oryzae*, *AMB Express*, **4**, 52, 52. doi: 10.1186/s13568-014-0052-9. eCollection 2014.
- Wong L.S., Khan F., Micklefield J., (2009), Selective covalent protein immobilization: strategies and applications, *Chemical Reviews*, **109**, 4025-4053.
- Yan T.R., Lin C.L., (1997), Purification and characterization of a glucose-tolerant beta-glucosidase from *Aspergillus niger* CCRC 31494, *Bioscience Biotechnology and Biochemistry*, **61**, 965-970.
- Yao J., Chen Q., Zhong G., Cao W., Yu A., Liu Y., (2014), Immobilization and characterization of tannase from a metagenomic library and its use for removal of tannins from green tea infusion, *Journal of Microbiology and Biotechnology*, **24**, 80-86.
- Yazdi M., Khosravi A., Nemati M., Motlagh N., (2003), Purification and characterization of two intracellular  $\beta$ -glucosidases from the *Neurospora crassa* mutant cell-1, *World Journal of Microbiology and Biotechnology*, **19**, 79-84.
- Zhou Y., Pan S., Wei X., Wang L., Liu Y., (2013), Immobilization of  $\beta$ -glucosidase onto magnetic nanoparticles and evaluation of the enzymatic properties, *Bioresources*, **8**, 2605-2619.