

RESEARCH ARTICLE



Improved octyl glucoside synthesis using immobilized β -glucosidase on PA-M with reduced glucose surplus inhibition

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ABSTRACT

A β -glucosidase extracted from bitter almond (*Prunus dulcis var. amara*) was immobilized on polyamine microspheres (PA-M) for catalytic octyl glucoside (OG) synthesis from glucose and octanol through reversed hydrolysis. The immobilization increased the activity of enzyme at pH 6.0–7.0, and the optimal reaction temperature for immobilized enzyme was identical to the free enzyme. The thermal stability and solvent tolerance of enzyme were increased by its immobilization. In the co-solvent system using 10% *t*-butyl alcohol and 10% (v/v) water, the yield of OG was increased by 1.7-fold compared to the yield from the system without co-solvent. Based on dynamic and Dixon plot analyses, the initial reaction velocity (V_0) increased approximately three-fold on immobilization and the OG synthesis was inhibited by surplus glucose. The inhibition dissociation constants for free and immobilized enzyme were 219 mM and 116 mM, respectively. A fed-batch mode was applied in the OG synthesis to minimize substrate inhibition. After 336 h of reaction, the OG yield and the conversion rate of glucose reached 134 mM and 59.6%, respectively. Compared to the batch operation, the fed-batch operation increased the OG yield and the conversion rate of glucose by 340% and 381%, respectively.

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Introduction

Alkyl glycosides belong to a family of low toxic biodegradable nonionic surfactants with properties of detergency, foaming, wetting and emulsification. They have potential applications in the food, cosmetic, pharmaceutical and detergent industries (Bhatia et al. 2002; Dembitsky 2004; Lalitha et al. 2015). Short-chain alkyl glucosides show a low level of surface activity, while the long-chain alkyl glucosides, such as octyl glucoside (OG), show significant surface activity (Lopez et al. 2001). OG has been used for solubilization (Rosevear et al. 1980), reconstitution of carrier (Newman et al. 1981) and crystallization of membrane proteins (Prive 2007).

Chemical methods for long-chain alkyl glycoside synthesis mainly rely on Fischer glycosylation and transacetalization (Corma et al. 1998). In the Fischer glycosylation, alcohol reacts directly with the sugar in the presence of catalysts to produce alkyl glycoside. In transacetalization, the carbohydrate is first coupled to

a short alcohol (usually butanol) producing the corresponding alkyl glycoside, which is subsequently transacetalated with a longer chain alcohol. Unfortunately, chemical procedures for the preparation of pure glycosides require multiple protection and deprotection steps increasing the cost of producing the long-chain alkylglucosides (Ducret et al. 2006). An alternative to chemical syntheses of glycosides is enzymatic synthesis relying on glycosidases that use mild reaction conditions and are regio- and stereo-selective (Rather and Mishra 2013).

β -Glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) is capable of catalysing the hydrolysis of β -glucosidic linkages in disaccharides and oligosaccharides and other glycoconjugates (Chang et al. 2013). β -Glucosidase can also be used in the synthesis of various glucosides by reverse enzymatic catalysis (Figure 1) (Van Rantwijk et al. 1999; Yu et al. 2008). The synthesis of alkyl glycosides catalysed by glycosidases can be carried out through the reversed hydrolysis and transglycosylation (Van Rantwijk et al. 1999).

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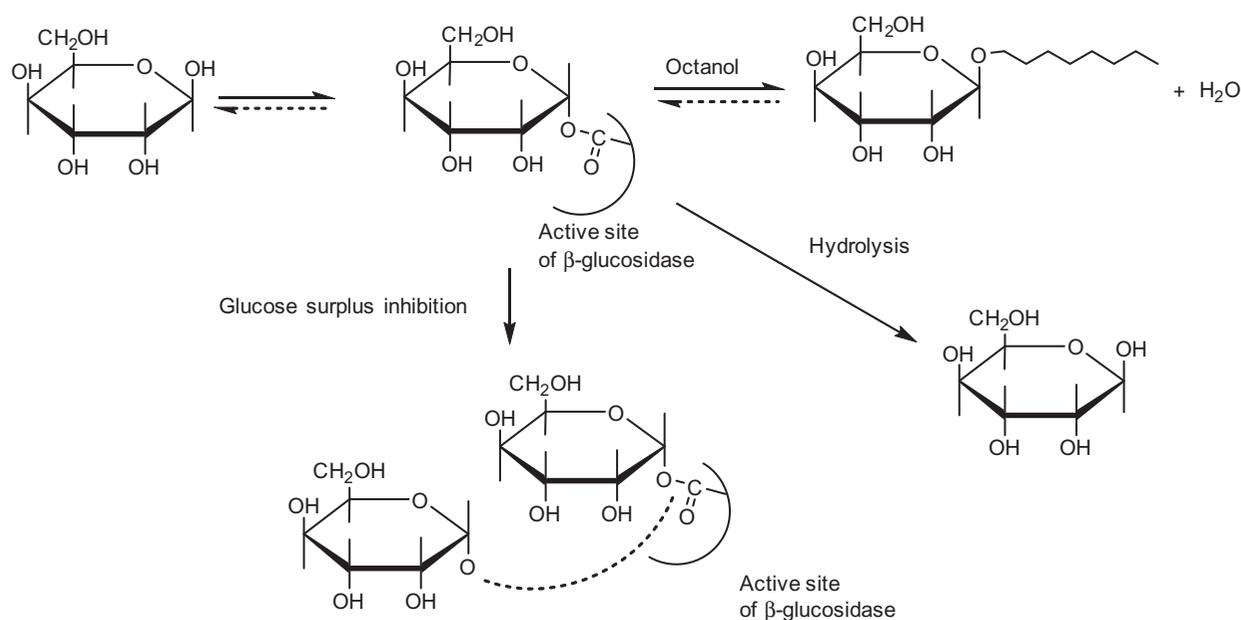


Figure 1. Reaction scheme of the conversion of glucose to octyl-β-glycoside (OG) in octanol (reversed hydrolysis) catalysed by β-glycosidase. Conversion of OG to glucose with water (hydrolysis reaction) was a side reaction. The secondary side reaction is the glucose inhibition on the OG synthesis.

Transglycosylation can give higher yields than the reversed hydrolysis does. However, reversed hydrolysis is more facile and economic by using monosaccharides and alcohols as the reactants or substrates and demands less complicated downstream processing for separating the by-products (Yang et al. 2015).

Glucosidases have been applied in the enzymatic synthesis of OG. Ducret et al. reported 13 glycosidases for the synthesis of OG and found almond β-glycosidase was less inhibited by high concentration of glucose (Ducret et al. 2002b). The rate of reaction and the percent conversion could be enhanced by using co-solvents, especially *N,N*-dimethylformamide (DMF) (Ducret et al. 2002a). The synthesis of OG was also examined through transglycosylation with almond β-glycosidase (Mladenoska 2016). The conversion rate reached 38%, which was higher than that (15.2%) obtained in reverse hydrolysis. However, the *p*-nitrophenyl-β-glucoside glucose donor used in transglycosylation is more expensive than the final product OG, thus, hampering its application.

Compared to the soluble enzymes, immobilized enzymes have the advantages of reusability and increased stability against inactivation (Wang et al. 2014). Various immobilization methods, such as linkage to solid supports, entrapment in hydrogels and micro-encapsulation, have been used for glucosidases from different sources (Ducret et al. 2006; Synowiecki and Wolosowska 2006; Wei et al. 2013). Enzyme stability is typically improved through immobilization. For example, the thermal stability of β-glycosidase

improved by its immobilization to Eupergit C resins (Tu et al. 2006). Polyethylene imine (PEI) was used to enhance the stability of β-glycosidase immobilized on agarose matrix (Vieira et al. 2011). Polyamine microspheres (PA-M), developed in our laboratory from PEI through emulsion polymerization, are microspheres with a high density of ionizable amino groups available to load enzymes through the electrostatic interactions (Wang et al. 2012, 2014). In this study, a β-glycosidase extracted from bitter almond was immobilized on PA-M. We measured the kinetic characteristics of this immobilized β-glycosidase for OG synthesis, thermal stability and solvent tolerance. A fed-batch mode was designed and investigated to reduce glucose inhibition of enzymatic OG synthesis.

Materials and methods

Materials

Bitter almond (*Prunus dulcis* var. *amara*) used for β-glycosidase extraction was a gift from Zhangye Chinese Medicine Technology Co., Ltd., Gangsu, China. 4-Nitrophenyl β-D-glucopyranoside (pNPG), *p*-nitrophenol (pNP), dimethyl sulphoxide (DMSO), DMF, *t*-butyl alcohol (*t*-BA), dimethoxyethane (DME), acetonitrile, DEAE52-cellulose, SephadexG-100, polyethyleneimine (Mw = 70,000 Da; 50 wt% solution in water) were purchased from Sigma-Aldrich, Shanghai Trading Co. Ltd. (Shanghai, China). Glutaraldehyde solution (25 wt%), liquid paraffin, Span-80 were obtained from

Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Extraction and purification of β -glucosidase

β -Glucosidase was extracted and purified from the fresh bitter almonds according to a previously described method (Yu et al. 2007). The fresh almonds were first peeled and powdered, washed three times with ethyl acetate, and two times with acetone at room temperature to remove lipid and then dried in a vacuum oven at 4 °C. Next, 1 g of defatted almond powder was soaked in 25 mL sodium phosphate buffer (50 mM, pH 7) for 2 h and centrifugation (10,000 $\times g$, 10 min). The supernatant was added with finely ground ammonium sulphate crystals to achieve 30% saturation to remove the impurity by centrifugation at 10,000 $\times g$ for 10 min. The precipitate was discarded and the supernatant was collected. By adding ground ammonium sulphate crystal, the saturation level of ammonium sulphate in the supernatant was increased to 70% to precipitate the crude enzyme. After centrifugation (10,000 $\times g$ for 10 min), the crude enzyme was collected and reconstituted in 50 mM sodium phosphate solution of pH 7 at the ratio of 1:1 (w/w). The ammonium sulphate in the enzyme solution was removed by dialysis at 4 °C overnight against the same buffer.

The enzyme solution obtained after dialysis was applied to a DEAE52-cellulose column (1.0 cm \times 20 cm) pre-equilibrated with 10 mM Tris-HCl buffer of pH 7. The elution was conducted with NaCl gradient from 0 M to 0.15 M at a flow rate of 0.5 mL/min.

Fractions containing β -glucosidase activity were collected and concentrated by ultrafiltration with Amicon[®] Ultra-15 centrifugal filter devices (Millipore of 10 kDa). The concentrated enzyme was further purified on a Sephadex G100 column (2.0 cm \times 100 cm) eluted with 10 mM pH 7.4 Tris-HCl buffer at a flow rate of 0.3 mL/min. The fractions possessing the highest activity of β -glucosidase were pooled and stored at 4 °C. Protein concentration was determined by the Bradford method (Bradford 1976) using bovine serum albumin as a standard and the enzyme purity and molecular weight were measured by SDS-PAGE (Laemmli 1970).

β -glucosidase activity assay

The hydrolytic activity of β -glucosidase was determined by measuring the release of *p*-nitrophenol (NPG) from *p*-nitrophenyl β -D-glucopyranoside (pNPG). All samples were assayed at 50 °C in potassium phosphate buffer (KPB, 50 mM, pH 7.0). Free enzyme (50 μ L)

or immobilized enzyme (20 mg) was allowed to react with 300 μ L pNPG (10 mM dissolved in KPB) for 120 s using 50 μ L of buffer as a control. The absorbance at 405 nm was measured with a microplate reader (Thermo Scientific[™], Waltham, MA). One unit of β -glucosidase hydrolysis activity was defined as the amount of enzyme that released 1.0 μ mol pNP/min at 50 °C.

Immobilization of β -glucosidase on PA-M microspheres

The preparation of the PA-M microspheres was conducted according to the method reported in our previous studies (Wang et al. 2012, 2014). PA-M (50 mg) was incubated with 2 mL β -glucosidase buffer solution (10 mg/mL) on a rotary shaker with an agitation speed of 500 rpm at 25 °C for 4 h. The adsorbed enzyme was separated by filtration and washed three-times with the same buffer to remove unbound enzyme. The β -glucosidase adsorbed PA-M was put into a 2 mL of 0.1 wt% glutaraldehyde solution (pH 7.5) and incubated for one hour on a rotary shaker (300 rpm, 25 °C). The immobilized β -glucosidase was collected by filtration and washed four-times with distilled water and stored at 4 °C. The coupling capacity of β -glucosidase on PA-M was 147 mg/g, and the specific activity of the immobilized enzyme was 2.01 U/mg protein.

The immobilization yield was defined as follows:

$$\text{Immobilization yield \%} = \left(\frac{a_{\text{imm}}}{a_{\text{free}}} \right) \times 100$$

where a_{imm} is the specific activity of immobilized enzyme (U/mg protein) and a_{free} is the specific activity of the initial enzyme preparation (U/mg protein).

The enzyme recovery of the process was calculated as the ratio of the total amount of bound enzyme to the total amount of enzyme initially present in the immobilization mixture.

Determination of enzyme kinetics parameters

The hydrolysis activity and kinetics of immobilized β -glucosidase were determined using pNPG as the substrate at 50 °C, pH 7.0. The Michaelis-Menten kinetic parameters of both free and immobilized β -glucosidase were determined from a Lineweaver-Burk plot.

In the β -glucosidase catalysed synthesis of OG, an excess amount of octanol was used compared to the amount of glucose, the kinetics of glucose inhibition on the OG synthesis could be described using the reduced Haldane equation (Sonnad and Goudar 2004). The kinetic parameters, inhibition dissociation constant (K_{SI}), maximal reaction velocity (V_{max}) and the

Michaelis–Menten constant (K_m), were determined from Dixon plots.

Influences of temperature and pH on the activity of immobilized β -glucosidase

The effect of temperature on the activity of β -glucosidase was determined at a pH 5.5 over temperatures ranging from 40 to 70 °C. The effect of pH on β -glucosidase activity was determined in 50 mM citric acid–citrate buffer (pH 4.0–6.0) and 50 mM sodium phosphate (pH 6.0–8.0) buffer. The relative activity (%) was calculated by comparing the activity of every sample with the maximum activity (defined as 100%). The data shown in the figures are average values and standard deviations of triplicated experiments.

Evaluation of thermal stability and solvent tolerance of β -glucosidase

The thermal stabilities of the free and immobilized β -glucosidase were tested by incubating the enzymes at 30–100 °C for 48 h in sodium phosphate buffer of pH 5.5. The enzymes were cooled in an ice bath and the residual activities were measured. The initial activity of the enzyme was defined as 100%.

The solvent tolerance of β -glucosidase was examined by incubating the enzyme at 50 °C in different co-solvent–octanol–water buffer systems. The water content in all tested systems was set at 15% (v/v total volume). The concentration of co-solvents, namely DMSO, DMF, t-BA, DME, and acetonitrile, was 2%, 5%, 10%, 20% and 30% (v/v total volume), respectively. Correspondingly, the final concentration of octanol was 83%, 80%, 75%, 65% and 55%, respectively. In the testing of the solvent tolerance of the free enzyme, 5 U β -glucosidase was added into a 1 mL of co-solvent–octanol–water buffer with various content of co-solvent. After incubation for 48 h, 50 μ L of enzyme solution was drawn, and the activity of enzyme was determined with pNPG as the substrate. For testing of the solvent tolerance of the immobilized enzyme, 20 mg immobilized enzyme was incubated in 1 mL of co-solvent–octanol–water buffer for 48 h. After that, the enzyme was separated and the activity was assayed. β -Glucosidase activity was expressed as % residual activity compared to the initial activity of the enzyme.

General procedure for enzymatic glucosylation

D-Glucose (0.5 mmol), octanol (10 mmol) and 0.1 mL t-BA were added into 0.3 mL of sodium phosphate

buffer (pH 6.0, 50 mM). The reaction, performed at 50 °C, was initiated by adding 5 U of enzyme. After 48 h of reaction, the reaction mixture was added to 10 mL methanol and filtered through a 0.22 μ m membrane. The amount of OG in the filtrate was determined by HPLC.

Influence of co-solvent addition on the OG synthesis catalysed by the immobilized enzyme

In a 2 mL of the reaction mixture, 0.5 mmol glucose and 0.2 mL of sodium phosphate buffer (pH 6.0, 50 mM), either DMSO, DMF, t-BA, DME, or acetonitrile were added together with octanol. The concentration of co-solvent was 2%, 5%, 10%, 20% and 30% (v/v total volume) with 15% water in octanol solution. Immobilized β -glucosidase (5 U) was added to initiate the reaction at 50 °C on a shaker with an agitation speed of 200 rpm. After 48 h, the reaction mixture was added with 10 mL methanol and was filtered with 0.22- μ m membrane. The amount of OG in the filtrate was measured and the concentration of OG in the reaction medium was calculated.

Influence of water content on the synthesis of OG

Glucose (0.5 mmol) and 0.1 mL t-BA were added to 1.9 mL mixture of sodium phosphate buffer (pH 6.0, 50 mM) and octanol. The effect of water content was investigated at the levels of 5%, 10%, 15%, and 20% (v/v total volume). The reaction was initiated by adding 5 U of enzyme and carried out at 50 °C for 48 h on a shaker at a speed of 200 rpm. After the reaction, 10 mL of methanol was added to the reaction mixture, which was then filtered through a 0.22- μ m membrane and the amount of OG in the filtrate was determined.

Synthesis of OG in a fed-batch mode

Based on the optimized reaction conditions of the batch operation, a glass reactor was designed for OG synthesis in fed-batch mode. The reactor with a working volume of 100 mL was comprised of a neck jacketed glass cylinder with three outlet ports, one for feeding the glucose powder, one connecting stirrer, and another for sampling. The temperature was maintained at 50 °C by circulating hot water through outer jacket. The reactor (with a volume of 80 mL) was filled with 64 mL of n-octanol, 12 mL of sodium phosphate buffer (50 mM, pH 6.0), 4 mL t-BA, 225 mM glucose and 200 U of enzyme. The glucose powder was fed every 6 h to keep the glucose concentration in the reactor nearly constant.

The feeding speed of glucose was determined according to following equation:

$$\frac{d(V_r C_s)}{dt} = F C_{S_0} - V_r M_t \quad (1)$$

where V_r is the volume of reaction mixture in the reactor (80 mL); C_s is the glucose concentration in the reactor (mM); C_{S_0} is glucose concentration in the feedings (5.55 mmol/g); F is the feeding speed of ground D-glucose powder (g); M_t is the consumption rate of glucose in the reactor (mM/h); t is the reaction time (h), assuming the addition of glucose powder had no effect on the volume of the reaction mixture and the glucose concentration remains constant upon reaching a pseudo-steady state. Therefore, Equation (1) can be reduced to be Equation (2).

$$F = V_r M_t \quad (2)$$

Samples were taken at regular intervals for the estimation of residual glucose and the yield of OG.

OG determination by HPLC

The quantity of OG was measured by HPLC equipped with an ODS column (YMC-Pack ODS-AQ Ø250 mm × 4.6 mm, 5 μm) and a refractive-index detector (model RID-10A; Shimadzu, Kyoto, Japan). HPLC was carried out at the room temperature using a methanol–water mixture (80:20, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The analytical errors were less than 1%.

Purification of OG (Ismail et al. 1999)

After evaporation of the t-TA and water under vacuum, OG in the reaction mixture was purified on a silica gel liquid chromatography column (70 × 3 cm, Merck, Kenilworth, NJ) using a dichloromethane–methanol solution (8:2, v/v) as the mobile phase at a flow rate of 4 mL/min.

Structure determination of OG by NMR and mass spectroscopy

^1H NMR of OG was recorded on Bruker FT DRX 500 (500 MHz). The chemical shifts were determined in parts per million (ppm) referenced to the appropriate solvent peak, methanol-d (MeOD), or to 0.0 ppm. The doublet at 4.24 ($J = 7.79$ Hz) in the ^1H NMR of purified OG confirmed β-configuration at the anomeric centre. ^1H NMR (MeOD): δ 4.24 (d, $J = 7.79$ Hz, 1H), 3.93–3.74 (m, 3H), 3.59 (dd, $J = 16.1, 7.4$ Hz, 1H), 3.48 (d, $J = 8.3$ Hz, 2H), 3.37 (s, 1H), 3.30 (t, $J = 8.0$ Hz, 1H), 1.65 (s, 2H), 1.31 (d, $J = 13.0$ Hz, 10H), and 0.88 (d, $J = 11.5$ Hz, 3H). The ^{13}C NMR spectrum recorded

14 carbon signals, including the carbon signals from glucose and octanol. ^{13}C NMR ((MeOD): δ 13.94 (C-8), 22.08 (C-7), 25.53 (C-3), 28.69 (C-4), 28.88 (C-5), 29.27 (C-2), 31.25 (C-6), 61.10 (C-6'), 68.53 (C-1), 70.10 (C-4'), 73.43 (C-2'), 76.79 (C-3', C-5'), and 102.83 (C-1'). 22.08, 25.53, 28.69, 28.88, 29.27, and 31.25 for CH₂-alkyl chain and 13.94 for alkyl CH₃. The peak at 102.83 (doublet) can be assigned to anomeric carbon. Mass spectra were recorded on a Bruker APEXIII 7.0TESL A FTMS (Billerica, MA) using ESI mode: m/z 315 (M + Na⁺) was detected for the molecular ions of OG.

Results and discussions

β-Glucosidase purification and immobilization

β-Glucosidase was extracted and purified from the fresh bitter almonds by two steps of ammonium sulphate precipitation, two steps of chromatography (DEAE column and Sephadex G100 column) and ultrafiltration. The overall β-glucosidase extraction and purification yield from bitter almonds was 0.456 U/g dry bitter almonds. The molecular weight of purified β-glycosidase was estimated to be 68 kDa by PAGE analysis (Figure 2).

PEI contains high density of amino functional groups and has been used in the immobilization of enzymes as the coating or building material of carriers for enzyme (Brady and Jordaan 2009). Microspheres of PA-M synthesized by cross-linking PEI through emulsion

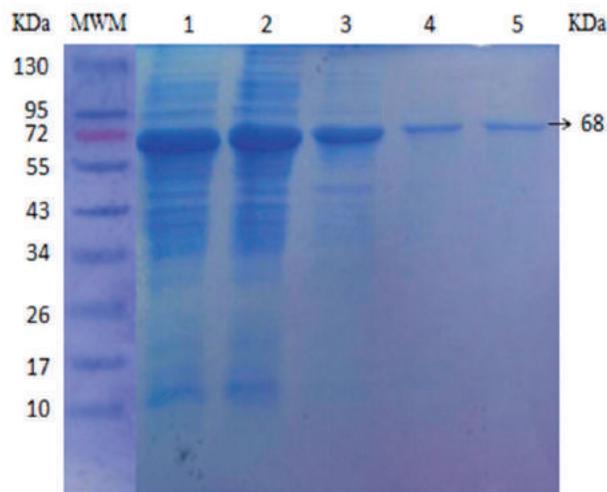


Figure 2. SDS-PAGE of β-glycosidase isolated from bitter almond during the process of purification (the lane on the far left is marker; lane 1, the crude extract; lane 2, the extract treated with 30% ammonium sulphate; lane 3, the crude enzyme precipitated by increasing the saturation level of ammonium sulphate in supernatant from 30% to 70%; lane 4, the enzyme obtained after purifying the crude enzyme on a DEAE52-cellulose column; lane 5, the final pure enzyme obtained using Sephadex G100 column purification).

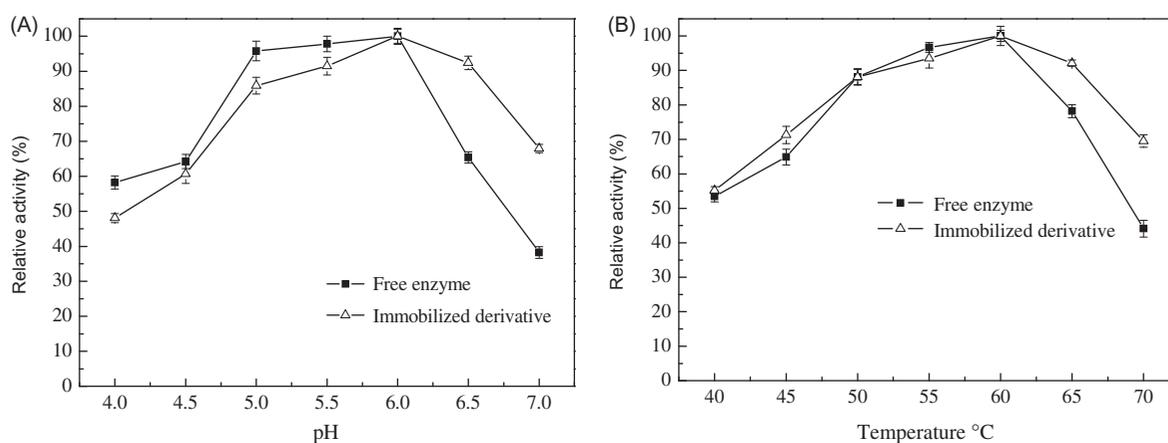


Figure 3. The hydrolysis activities of free β -glucosidase and the PA-M immobilized β -glucosidase under various values of pH (A) and temperature (B).

polymerization have the diameter distribution in the range of 0.37–4.29 μm (Figure S1). Analysis of the surface charge of PA-M using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY) demonstrated the isoelectric point (pI) of the microspheres was 10.6. Element analysis using a Vario EL III elemental analyser (Elemental Analysis, Langensfeld, Germany) demonstrated the N content of the microspheres was $16.58 \pm 0.15\%$ (wt%). Enzyme was first incubated with PA-M at neutral pH to be physically adsorbed on the microspheres. Electrostatic attraction between the amino groups of PA-M and the charged β -glucosidase (pI 4.3) together with hydrogen bonds and Van der Waals forces drive this adsorption. The adsorbed enzyme was then treated with glutaraldehyde under mild conditions. This results in cross-linking between the amino groups of the enzyme and the amino groups of the support establishing multi-point covalent enzyme-support attachment. Under the given conditions of immobilization, the immobilization yield reached 84.8% and the enzyme recovery was 58.4%.

Optimal pH and temperature for the hydrolysis activity of the free and immobilized β -glucosidase

The activity of β -glucosidase is in direct relationship with the specific protonation status of catalytic amino acids (glutamate or aspartate) in the active sites of the enzyme, which is influenced by pH (Woodward and Arnold 1981). The pH-activity profiles of the free β -glucosidase and the immobilized β -glucosidase at 50 °C are shown in Figure 3(A). The relative activity of both free and immobilized enzyme increased with increasing pH and reaching an optimum value at pH 6.0. These activities sharply decreased when the pH exceeded 7.0. The relative activities of enzyme decreased after immobilization in the pH range of 4.0–6.5. However, in the range of pH 6.0–7.0, the immobilized enzyme maintained a

higher relative activity than the free enzyme. This may be due to the protonation of amino groups of PA-M constructing suitable microenvironments around the bound enzyme molecules (Akinc et al. 2005).

The effect of temperature, on the activity of the free and immobilized β -glucosidases, was examined at pH 6.0 over temperatures ranging from 40 °C to 70 °C (Figure 3(B)). The activities of both free and immobilized enzymes increased with increased temperatures below 60 °C. The activities of both enzymes reached a maximum at 60 °C. Thus, the optimal temperature for β -glucosidase was unchanged after immobilization. The immobilized enzyme retained about 70% of its initial activity, while the free enzyme only retained about 40% of its initial activity at 70 °C (Figure 3(B)). This suggests that immobilization protects β -glucosidase from the thermal deactivation. Besides, the enhancing enzyme activity, high temperatures may also thermodynamically increase the reaction velocity.

The slopes of the temperature–activity profiles (Figure 4), within the region where temperature dependent denaturation did not occur (Figure 3(B)), were used to calculate the activation energy of the free and immobilized enzyme mediated reaction using the Arrhenius equation (Wang et al. 2014). The activation energy was 3.45 kJ/mol for the free enzyme and 3.06 kJ/mol for the immobilized derivative. The slightly reduced activation energy of the reaction catalysed by immobilized β -glucosidase implied that the active conformations of the enzyme molecules were probably maintained during the immobilization.

Thermal stability and organic solvents tolerance of β -glucosidase

Next, the thermal stability and organic solvent tolerance of β -glucosidase were investigated. The

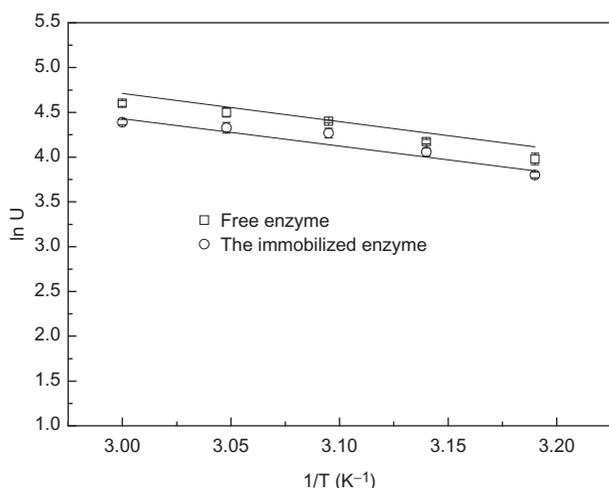


Figure 4. Arrhenius plots for the free enzyme (□) and the immobilized derivative (○). The slope of a logarithmic Arrhenius plot is related to the activation energy (E_a) for the molecule by the relationship: slope = $-E_a/R$, where R (8.314 J/mol/K) is the gas constant.

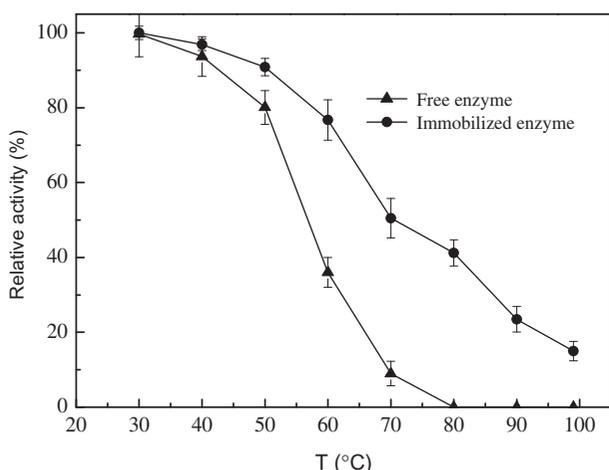


Figure 5. Thermal stability of the free and immobilized β -glucosidase.

immobilized enzyme retained about 40% of its initial activity after incubating at 80 °C for 48 h while the free β -glucosidase almost lost all of its activity (Figure 5). The results suggest that the thermal stability of β -glucosidase increased on immobilization. Other carriers for β -glucosidase immobilization, such as chitosan and Eupergit C resin, were also found to enhance enzyme thermal stability (Tu et al. 2006).

The commonly used glycone in alkyl glucoside synthesis, catalysed by glycosidase through reversed hydrolysis, is carbohydrate like glucose, which is very soluble in water but nearly insoluble in organic solvents, especially for long-chain alkyl glucoside synthesis. In contrast, aglucones, such as octanol used in OG synthesis, are poorly soluble in water. Thus, the enzymatic synthesis of OG using reversed hydrolysis is

challenging due to the poor miscibility of octanol in water and the low solubility of glucose in organic media. One approach for promoting long-chain alkyl glucoside synthesis is to increase the miscibility of glycone and aglycone in the reaction media by adding a water-miscible solvent as the co-solvent. For instance, Ducret et al. found the reaction rate and the yield of OG synthesis, catalysed by immobilized almond β -glucosidase, could be enhanced by using acetonitrile and DMF co-solvents (Ducret et al. 2002a). However, a limiting factor for application of co-solvent in enzyme-mediated alkyl glucoside synthesis is the low activity and stability of enzymes in non-aqueous environments due to the decreased water activity (Ducret et al. 2002a). The tolerance of β -glucosidase to the different contents of co-solvents were tested here by determining the activity of enzyme after incubating the enzyme in the co-solvent–octanol–water system at 50 °C for 48 h and the results are presented in Figure 6. The selected co-solvents, DMSO, DMF, t-BA, and DME, are organic co-solvents that have been reported to be widely used in the non-aqueous system of enzymatic catalysis for enhancing the miscibility of solvents and buffers (Karagulyan et al. 2008). Free enzyme could maintain 54% of its activity after incubating in the pure octanol (Figure 6). Enzyme activity decreased as the concentration of added co-solvents increased. DMSO and DMF addition had the greatest negative impact on the activity of the free β -glucosidase. The free enzyme lost all of its activity, in a co-solvent–octanol–water buffer system, when the concentration of DMSO and DMF addition was >2% (v/v). The highest value of residual relative activity (48%) for the free enzyme was observed in the case of t-BA addition. The immobilization with PA-M as the support significantly increased the stability of β -glucosidase in the presence of co-solvent based on the results in Figure 6(B). The highest residual activity of the immobilized enzyme was demonstrated in the system using t-BA as the co-solvent. Approximately 80% residual relative activity could be retained after 48 h of incubation in the medium containing 2–10% t-BA. It is believed the polarity of organic solvent used in bio-catalysis has a direct relation with the activity of enzyme, and the logarithm of the partition coefficient, $\log P$, has been proposed as a quantitative measure of solvent polarity (Dordick and Isermann 2009). Hydrophilic solvent ($\log P < 2$) can strip away the water associated with the enzyme resulting in catalytic deactivation. The value of $\log P$ of octanol is 2.9 and other co-solvents show much lower $\log P$ values, i.e. -1.3 (DMSO), -1.0 (DMF), -0.33 (acetonitrile), -0.2 (DME), and 0.584 (t-BA), respectively. Owing to their low values of $\log P$, the

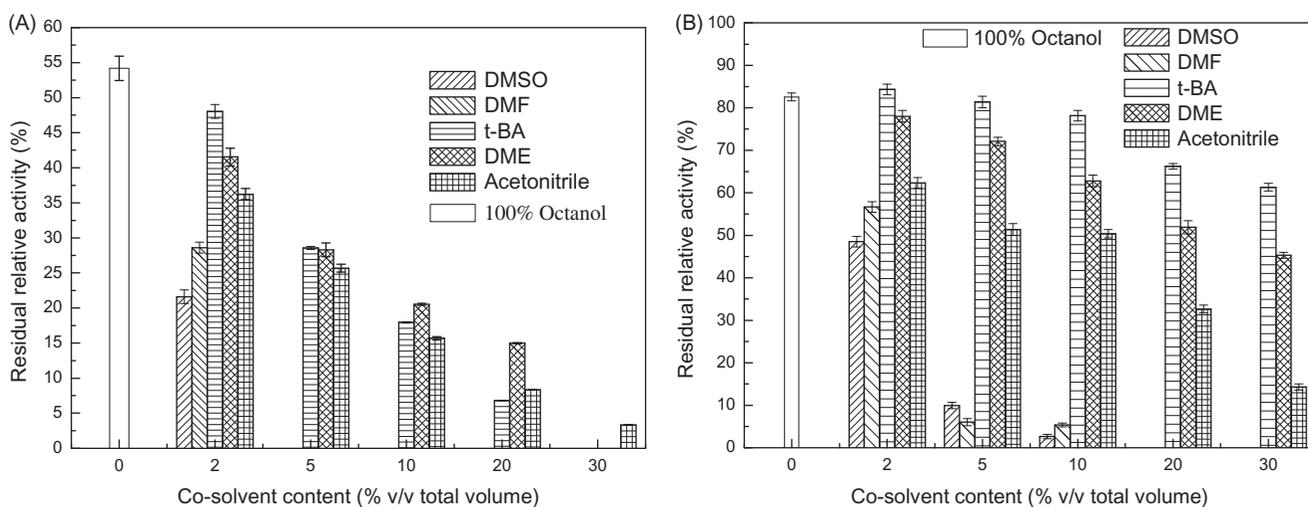


Figure 6. Organic solvent tolerance of the free (A) and immobilized β -glucosidase (B).

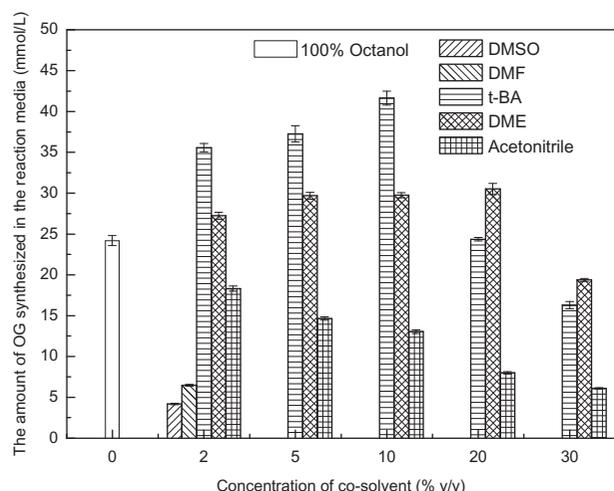


Figure 7. Effect of co-solvent content on the OG yield in the synthesis catalysed by immobilized β -glucosidase.

activity of β -glucosidase was reduced maximally in the media containing DMSO or DMF as the co-solvent. The log P value of t-BA was the highest among the co-solvents tested which accounts for the highest activities of the free enzyme and the immobilized derivative in the system with t-BA as the co-solvent.

The effect of different co-solvents addition on OG synthesis

The use of an organic co-solvent in the enzymatic synthesis of alkyl glucosides allows the reaction equilibrium to shift towards the synthesis reaction (Figure 1) rather than towards hydrolysis reaction, as a result of decreasing the water concentration (Selisko et al. 1990). The effect of the addition of different co-solvents on OG synthesis is shown in Figure 7. The addition DMF and DMSO (2%) greatly impaired the OG

synthesis, reducing the yield by 83% and 73%, respectively. The OG synthesis was terminated when the concentration of DMF or DMSO was higher than 2%. The yields of OG synthesized by immobilized β -glucosidase were improved by t-BA and DME in addition over the concentration range of 2–10% and 2–20%, respectively. The most pronounced effect was found in the case of 10% t-BA addition. In this case, the yield of OG increased by 1.7-fold compared to the yield obtained in the synthesis conducted in reaction media not containing co-solvent. The application of co-solvent to construct low water environments, facilitating the synthesis of glycosides catalysed by glycosidases through reversed hydrolysis, has attracted considerable interest in recent years. For instance, Ducret et al. found adding 20% DMF in the reaction media of OG synthesis catalysed by almond β -glucosidase can increase OG yield by 2.5-fold (Ducret et al. 2002a).

Influence of water content on OG synthesis by the immobilized enzyme

In the synthesis of alkyl glucosides catalysed by glycosidases through reversed hydrolysis, water acts as a competing nucleophile and causes parasitic hydrolysis of the product (Van Rantwijk et al. 1999). It is clear that a lower water concentration in media favours the equilibrium yield of glycoside product. While application of co-solvent can improve the miscibility of aglycone and glycone and decrease the water demand for the solubility of glucose in the medium for OG synthesis, a minimum amount of water is necessary to maintain the enzyme activity (Vic et al. 1995). In addition, the position of the reaction equilibrium greatly depends on water content of the medium. The yields of OG synthesis catalysed by β -glucosidase

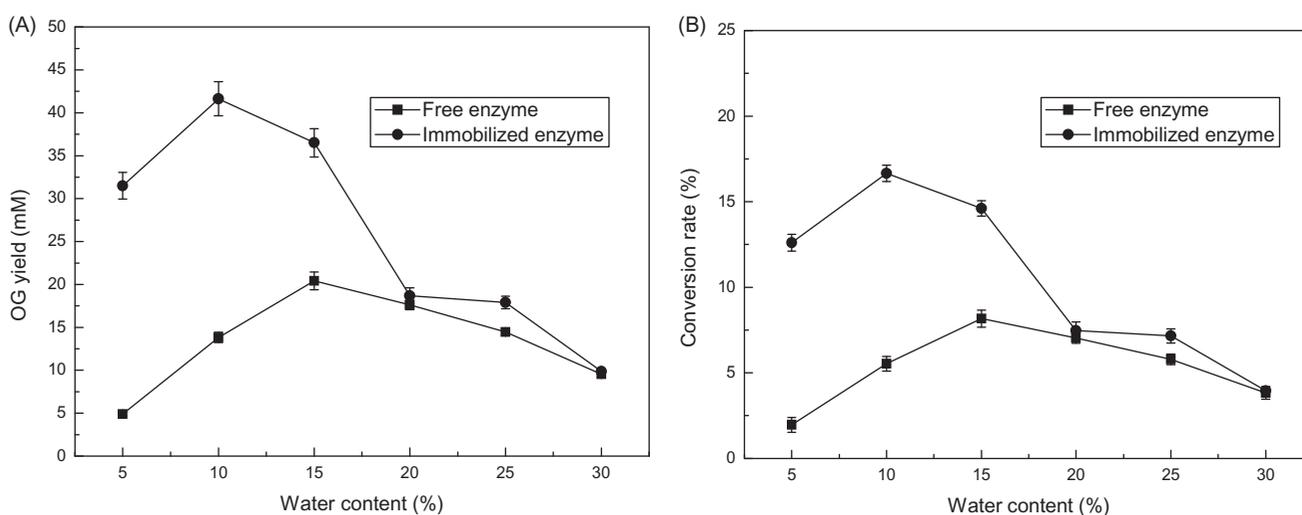


Figure 8. Influence of various water contents in the medium on the yield of OG synthesis catalysed by PA-M immobilized β -glucosidase (the initial concentration of glucose was 0.25 mM).

immobilized on PA-M in the media of different water content were presented in Figure 8. The OG yields and the conversion rates of glucose can be increased by more than three-fold after the immobilization of β -glucosidase. The maximal value of OG yield in the synthesis catalysed by the immobilized enzyme was obtained in the medium of 10% (v/v) water content, which was less than that (15%) for obtaining the maximal yield using the free enzyme. This may be because the immobilization increased the organic solvent tolerance of β -glucosidase reducing the amount of water needed for the maintenance of enzymatic activity during the OG synthesis. Reducing the water content further to 5% and 10%, the yield and the conversion rate of OG synthesis did not increase because the activity of enzyme is optimal at certain water content and the less water content may be unfavourable for the enzyme to maintain its proper active configuration. Changing the water content can alter the water activity in the medium of the water immiscible solvent favouring the synthesis of alkyl glucosides (De Roode et al. 2001). However, the alkyl glucosides are surface active substances which can interfere with the interaction between water and water in the reaction media (Mathlouthi 2001), thus, how to control the water activity during the reaction is the main objective of our future research.

Influence of glucose concentration on the OG synthesis catalysed by immobilized β -glucosidase

Based on the nature of reversed hydrolysis, the yield of OG should be increased with increased glucose concentration, pushing the equilibrium towards product formation (Panintrarux et al. 1995). The effect of

increased initial glucose concentrations on the OG synthesis reaction yield and the conversion rate, catalysed by the immobilized β -glucosidase and the free enzyme at 50 °C in the media with 10% t-BA as the co-solvent, is presented in Figure 9. The conversion rate of glucose to OG was reduced at increased glucose concentrations (Figure 9). The maximal yield of OG catalysed by the immobilized enzyme, about 40.8 mM, was obtained when the content of glucose in the medium was 250 mM. This value of yield was 2.9-fold higher than the highest value of yield catalysed by the free enzyme obtained at the glucose concentration of 150 mM. The yield of OG decreased as the glucose concentration >250 mM in the case of immobilized enzyme and >150 mM when the free enzyme was used in the OG synthesis. These results suggest that surplus glucose inhibited OG synthesis.

Glucose inhibition dynamic analysis on the OG synthesis catalysed by β -glucosidase

Dynamic analysis (Figure 10) was conducted to understand the relationship between the initial reaction velocity (V_0) and the glucose concentration in OG synthesis catalysed by free and immobilized β -glucosidase at 50 °C. In non-aqueous medium, containing 10% t-BA as the co-solvent, the V_0 value of OG synthesis increased with increased glucose concentration and reached at the maximum value at approximately 200 mM of glucose. The maximum value of V_0 increased about three-fold by enzyme immobilization, from 0.40 mM/h to 1.23 mM/h (Figure 10(A)). The velocity of OG synthesis decreased when the glucose concentration increased to >200 mM. The Dixon plots of the initial reaction velocity against the

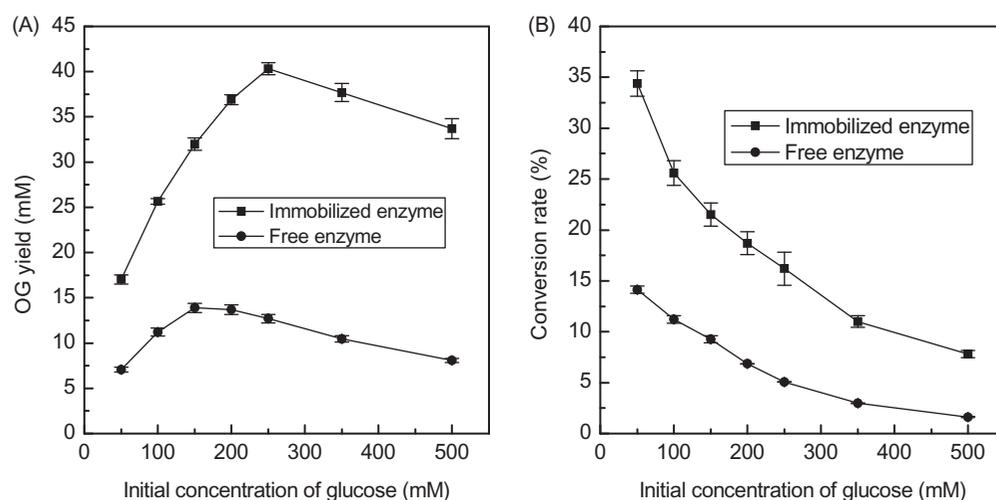


Figure 9. OG synthesis using immobilized β -glucosidase at different initial concentration of glucose at 50 °C with 10% t-BA as the co-solvent.

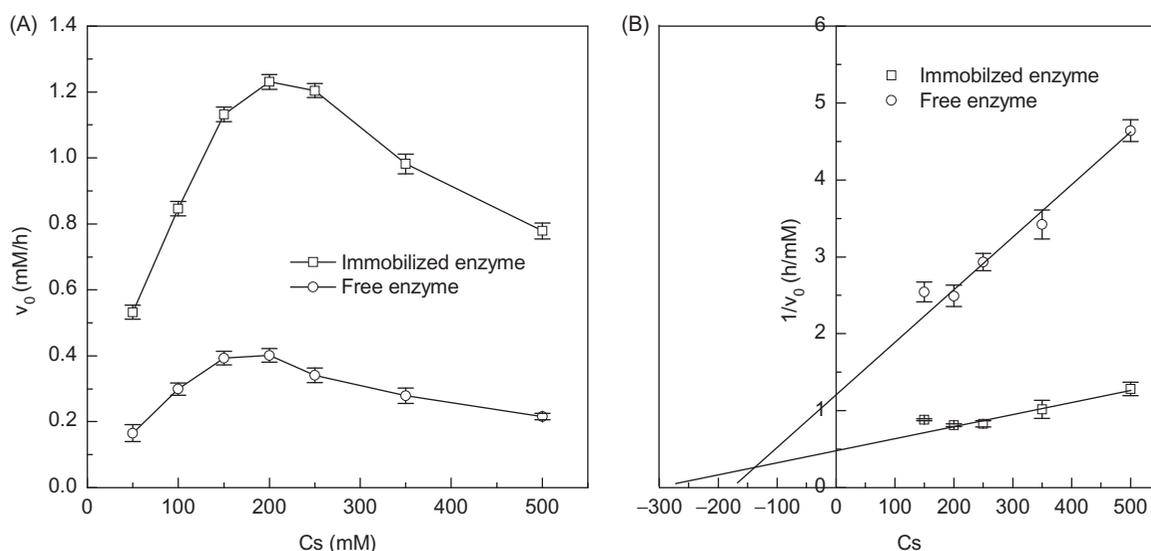


Figure 10. Influence of various glucose concentrations on the initial reaction velocity (A) and Dixon plots (B) of the OG synthesis by the free β -glucosidase and immobilized derivative on PA-M.

concentration of glucose clearly indicated that the V_0 of OG was inhibited at high glucose concentrations (Figure 10(B)), and that the inhibition was predominantly non-competitive (Stambaugh and Post 1966). According to the Haldane equation, the predicted maximal value of V_0 appears at 225 mM glucose concentration for the immobilized enzyme and 180 mM glucose concentration for the free enzyme. The value is close to the glucose concentration, 250 mM (the immobilized enzyme) and 150 mM (the free enzyme), respectively, used for obtaining the highest yield of OG (Figure 9). Based on the Dixon plots (Figure 10(B)), the value of inhibition dissociation constant (K_{SI}) was 219 mM for the free enzyme and 116 mM for the immobilized enzyme. Hence, the affinity of

β -glucosidase to glucose increased after enzyme immobilization. The results of simulation with Haldane equation showed the optimal glucose concentration for obtaining maximal V_0 using the free enzyme and the immobilized derivative was 180 mM and 225 mM, respectively. The immobilization reduced the effect of substrate inhibition on the OG synthesis catalysed by β -glucosidase. The substrate inhibition is non-competitive inhibition which occurs in about 20% of all known enzymes (Ekinci and Senturk 2010). This is in agreement with a recent report describing a weak glucose inhibition on almond β -glucosidase (Reshmi and Sugunan 2013). In this study, an obvious glucose inhibition was found in the OG synthesis catalysed by the β -glucosidase extracted from bitter almonds.

OG synthesis in the fed-batch mode

The glucose concentration should be kept at a reasonable level during the whole reaction period to eliminate/reduce the substrate inhibition and maintain the reaction velocity as high as possible. Strategies to overcome substrate inhibition include feeding of substrate to maintain its concentration below a certain limit (fed-batch reaction) or application of a continuous stirred tank reactor (CSTR) to minimize substrate concentration in the reactor (Bommarius and Riebel-Bommarius 2004). However, using a CSTR can cause catalyst loss in the outflow. In this study, the fed-batch reaction mode was used to control the glucose concentration in reactor to minimize the glucose inhibition. According to the results of batch reaction of OG synthesis, the reaction velocity of OG synthesis catalysed by the immobilized enzyme and the free enzyme reached maximal value of 1.23 mM/h and 0.40 mM/h at 225 mM and 180 mM of glucose concentration. Ground powder of glucose was fed in every 6 h to maintain the glucose concentration in the reactor to obtain a high reaction velocity. The feeding rate of glucose was determined by its consumption rate during reaction. The loss rate of enzyme activity in the longer term of incubation was also considered in determining the feeding rate of glucose. In a batch mode, the immobilized β -glucosidase lost about 57.4% of its initial activity in 306 h while the free enzyme maintained 27.3% of its initial activity in 48 h (data not shown). The half-life value of the immobilized enzyme and the free enzyme was 357 h and 25.6 h separately. The deactivation constant (K_d) for the immobilized enzyme and the free enzyme was 0.0028 h^{-1} and 0.027 h^{-1} , respectively. Therefore, the activity of immobilized enzyme at t time of reaction can be determined by calculation based on the equation:

$$C_E = C_{E0} \exp(-At) \quad (3)$$

where C_E is the activity of enzyme at t time, C_{E0} is the initial activity of enzyme. A is 0.0028 h^{-1} in the case of immobilized enzyme and 0.027 h^{-1} in the case of free enzyme.

Assuming the glucose consumption rate (M_t) was in linear relation to the activity change of the immobilized enzyme, the change of M_t with the reaction time can be determined by Equation (4):

$$M_t = M_0 \exp(-At) \quad (4)$$

where M_t is the consumption rate of glucose at the reaction time of t (mM/h), M_0 is the maximal consumption rate of glucose, which value equals to the value of V_{max} (1.23 mM/h for immobilized enzyme and 0.40 mM/h for the free enzyme).

When the fed batch operation reached a pseudo-steady state, the glucose concentration remains constant and close to zero. Combining Equations (2) and (4), Equation (5) was obtained for determining the feeding rate of glucose.

$$F = 80 \times M_0 \exp(-At) \quad (5)$$

The results of fed-batch reaction of OG synthesis catalysed by β -glucosidase immobilized on PA-M are shown in Figure 11(A). The fed-batch reaction lasted for 288 h, after which it was changed to a batch reaction. In the course of the fed-batch reaction, the concentration of glucose in the reactor could be maintained at approximately 225 mM with the regular addition of fine glucose powder. A 225 mM of glucose concentration in the reaction should be optimal for achieving the maximum reaction velocity of OG synthesis (Figure 10). At the end of 336 h of reaction, the OG yield and the conversion rate of glucose were 134 mM and 59.6%, respectively. Compared to the batch reaction, the fed-bath reaction was able to increase the OG yield and the conversion rate of glucose by 340% and 381%, respectively. In the case with free enzyme, a fed-batch reaction was carried out for 72 h, after that it was changed to a batch reaction. According to Figure 10(B), a 180 mM of glucose concentration in the reaction should be optimal for achieving the maximum reaction velocity of OG synthesis. At the end of 96 h of reaction, the OG yield and the conversion rate of glucose were 43.58 mM and 20.7% (Figure 11(B)). Compared to the batch reaction, the fed-bath reaction was able to increase the OG yield and the conversion rate of glucose by about 288% and 246.7%. Unlike in the case with the immobilized enzyme, the batch reaction carried out after the fed-batch reaction with the free enzyme produced a small amount of OG. This may be due to the lower stability of the free enzyme caused by denaturation after 72 h of fed-batch reaction. Hence, the immobilization on PA-M can significantly increase the output of the OG synthesis catalysed by the β -glucosidase through enhanced stability of the enzyme.

The yield and the conversion rate of OG synthesis mediated by the almond β -glucosidase immobilized on PA-M using reversed hydrolysis in fed-batch mode of reaction was higher than that obtained using transglycosylation catalysed by almond β -glucosidase adsorbed onto Celite R-640 or by yeast *Pichia etchellsii* displaying cell wall bound β -glucosidase using pNPG as glycone (Basso et al. 2002; Rather et al. 2010). The fed-batch mode of reaction is simple and easy to be scaled up. Compared to batch operation, fed-batch operation increases both the yield and conversion rate

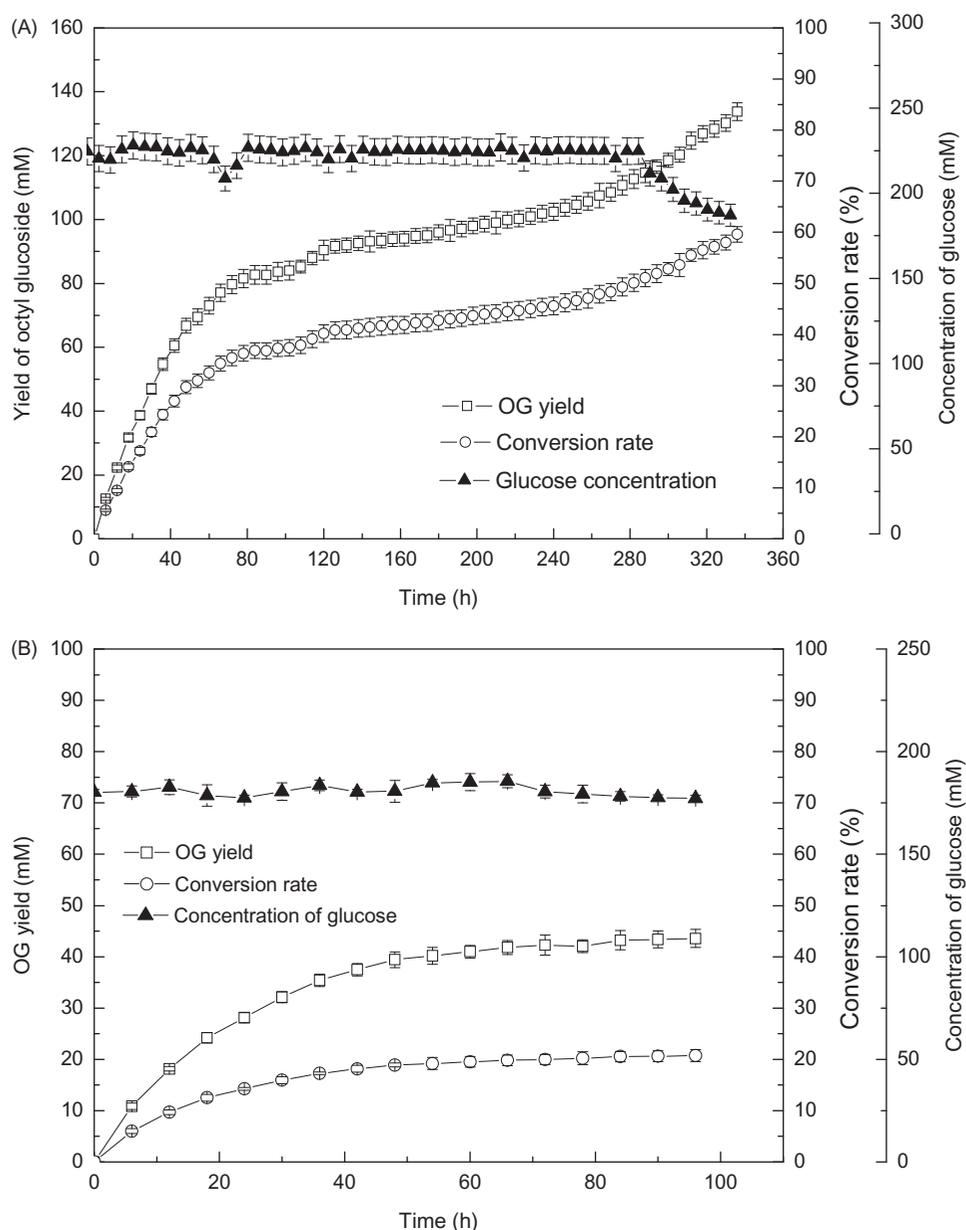


Figure 11. Time course of OG synthesis catalysed by the β -glucosidase immobilized on PA-M (A) and the free enzyme (B) in the fed-batch reaction mode.

of OG synthesis by dilution of glucose inhibition. That will lower down-stream processing cost due to higher product concentration.

Conclusions

The OG synthesis mediated by β -glucosidase using reversed hydrolysis can be promoted by the immobilization. The OG yield was increased by 1.7-fold when 10% t-BA was used as co-solvent in the reaction medium. The OG yield and the initial reaction velocity reduced when the concentration of glucose was >225 mM. Therefore, a fed-batch mode was used to minimize the glucose inhibition and maintain the

reaction velocity of OG synthesis as high as possible during the OG synthesis. The OG yield and the conversion rate of glucose increased 340% and 381% by using a fed-batch reaction.

Disclosure statement

The authors have no conflict of interest and were solely responsible for the writing and content of the paper.

References

Akinc A, Thomas M, Klivanov AM, Langer R. 2005. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J Gene Med* 7:657–663.

- Basso A, Ducret A, Gardossi L, Lortie R. 2002. Synthesis of octyl glucopyranoside by almond β -glucosidase adsorbed onto Celite R-640[®]. *Tetrahedron Lett* 43:2005–2008.
- Bhatia Y, Mishra S, Bisaria VS. 2002. Microbial β -glucosidases: cloning, properties, and applications. *Crit Rev Biotechnol* 22:375–407.
- Bommarius AS, Riebel-Bommarius BR. 2004. *Biocatalysis: fundamentals and applications*. Weinheim: John Wiley & Sons.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Brady D, Jordaan J. 2009. Advances in enzyme immobilisation. *Biotechnol Lett* 31:1639–1650.
- Chang J, Lee Y-S, Fang S-J, Park D-J, Choi Y-L. 2013. Hydrolysis of isoflavone glycoside by immobilization of β -glucosidase on a chitosan-carbon in two-phase system. *Int J Biol Macromol* 61:465–470.
- Corma A, Iborra S, Miquel S, Primo J. 1998. Preparation of long-chain alkyl glucoside surfactants by one-step direct Fischer glucosidation, and by transacetalation of butyl glucosides, on Beta Zeolite catalysts. *J Catal* 180:218–224.
- De Roode BM, Van Beek J, Van Der Padt A, Franssen MCR, Boom RM. 2001. The integrated enzymatic production and downstream processing of hexyl glucoside. *Enzyme Microb Technol* 29:513–520.
- Dembitsky VM. 2004. Astonishing diversity of natural surfactants: 1. Glycosides of fatty acids and alcohols. *Lipids* 39:933–953.
- Dordick JS, Isermann HP. 2009. Rules for optimization of biocatalysis in organic solvents introduction. *Biotechnol Bioeng* 102:1–1.
- Ducret A, Carriere J-F, Trani M, Lortie R. 2002a. Enzymatic synthesis of octyl glucoside catalyzed by almond β -glucosidase in organic media. *Can J Chem* 80:653–656.
- Ducret A, Trani M, Lortie R. 2002b. Screening of various glucosidases for the synthesis of octyl glucoside. *Biotechnol Bioeng* 77:752–757.
- Ducret A, Trani M, Lortie R. 2006. Comparison between various commercial sources of almond β -glucosidase for the production of alkyl glucosides. *J Mol Catal B Enzym* 38:91–94.
- Ekinci D, Senturk M. 2010. Interactions of fungicides and pesticides with specific enzymes. In: *Fungicides*. In-Tech 383–404.
- Ismail A, Soultani S, Ghoul M. 1999. Enzymatic-catalyzed synthesis of alkylglycosides in monophasic and biphasic systems. II. The reverse hydrolysis reaction. *J Biotechnol* 69:145–149.
- Karagulyan HK, Gasparyan VK, Decker SR. 2008. Immobilization of fungal β -glucosidase on silica gel and kaolin carriers. *Appl Biochem Biotechnol* 146:39–47.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lalitha K, Muthusamy K, Prasad YS, Vemula PK, Nagarajan S. 2015. Recent developments in β -C-glycosides: synthesis and applications. *Carbohydr Res* 402:158–171.
- Lopez O, Cocera M, Parra JL, De La Maza A. 2001. Influence of the alkyl chain length of alkyl glucosides on their ability to solubilize phosphatidylcholine liposomes. *Colloids Surf A: Physicochem Eng Asp* 193:221–229.
- Mathlouthi M. 2001. Water content, water activity, water structure and the stability of foodstuffs. *Food Control* 12:409–417.
- Mladenoska I. 2016. Synthesis of octyl- β -glucoside catalyzed by almond β -glucosidase in unconventional reaction media. *Food Technol Biotechnol* 54:211–216.
- Newman MJ, Foster DL, Wilson TH, Kaback HR. 1981. Purification and reconstitution of functional lactose carrier from *Escherichia coli*. *J Biol Chem* 256:11804–11808.
- Panintrarux C, Adachi S, Araki Y, Kimura Y, Matsuno R. 1995. Equilibrium yield of n-alkyl- β -d-glucoside through condensation of glucose and n-alcohol by β -glucosidase in a biphasic system. *Enzyme Microb Technol* 17:32–40.
- Prive GG. 2007. Detergents for the stabilization and crystallization of membrane proteins. *Methods* 41:388–397.
- Rather MY, Mishra S, Chand S. 2010. β -Glucosidase catalyzed synthesis of octyl- β -d-glucopyranoside using whole cells of *Pichia etchellsii* in micro aqueous media. *J Biotechnol* 150:490–496.
- Rather MY, Mishra S. 2013. β -Glycosidases: an alternative enzyme based method for synthesis of alkyl-glycosides. *Sustain Chem Process* 1:1.
- Reshmi R, Sugunan S. 2013. Improved biochemical characteristics of crosslinked β -glucosidase on nanoporous silica foams. *J Mol Catal B Enzym* 85–86:111–118.
- Rosevear P, VanAken T, Baxter J, Ferguson-Miller S. 1980. Alkyl glycoside detergents: a simpler synthesis and their effects on kinetic and physical properties of cytochrome c oxidase. *Biochemistry* 19:4108–4115.
- Selisko B, Ulbrich R, Schellenberger A, Muller U. 1990. Invertase-catalyzed reactions in alcoholic solutions. *Biotechnol Bioeng* 35:1006–1010.
- Sonnad JR, Goudar CT. 2004. Solution of the Haldane equation for substrate inhibition enzyme kinetics using the decomposition method. *Math Comput Model* 40:573–582.
- Stambaugh R, Post D. 1966. Substrate and product inhibition of rabbit muscle lactic dehydrogenase heart (H4) and muscle (M4) isozymes. *J Biol Chem* 241:1462–1467.
- Synowiecki J, Wolosowska S. 2006. Immobilization of thermostable β -glucosidase from *Sulfolobus shibatae* by cross-linking with transglutaminase. *Enzyme Microb Technol* 39:1417–1422.
- Tu M, Zhang X, Kurabi A, Gilkes N, Mabee W, Saddler J. 2006. Immobilization of beta-glucosidase on Eupergit C for lignocellulose hydrolysis. *Biotechnol Lett* 28:151–156.
- Van Rantwijk F, Woudenberg-van Oosterom M, Sheldon RA. 1999. Glycosidase-catalysed synthesis of alkyl glycosides. *J Mol Catal B: Enzym* 6:511–532.
- Vic G, Biton J, Le Beller D, Michel J-M, Thomas D. 1995. Enzymatic glucosylation of hydrophobic alcohols in organic medium by the reverse hydrolysis reaction using almond-beta-d-glucosidase. *Biotechnol Bioeng* 46:109–116.
- Vieira MF, Vieira AMS, Zanin GM, Tardioli PW, Mateo C, Guisan JM. 2011. β -Glucosidase immobilized and stabilized on agarose matrix functionalized with distinct reactive groups. *J Mol Catal B: Enzym* 69:47–53.
- Wang F, Liu P, Nie T, Wei H, Cui Z. 2012. Characterization of a polyamine microsphere and its adsorption for protein. *Int J Mol Sci* 14:17–29.
- Wang F, Nie TT, Shao LL, Cui Z. 2014. Comparison of physical and covalent immobilization of lipase from *Candida*

- antarctica* on polyamine microspheres of alkylamine matrix. *Biocatal Biotransform* 32:314–326.
- Wei C, Lu Q, Ouyang J, Yong Q, Yu S. 2013. Immobilization of β -glucosidase on mercaptopropyl-functionalized mesoporous titanium dioxide. *J Mol Catal B: Enzym* 97:303–310.
- Woodward J, Arnold SL. 1981. The inhibition of β -glucosidase activity in *Trichoderma reesei* C30 cellulase by derivatives and isomers of glucose. *Biotechnol Bioeng* 23:1553–1562.
- Yang R, Wang Z, Bi Y, Jia J, Zhao X, Liu X, Du W. 2015. Convenient synthesis of alkyl and phenylalkyl β -d-glucopyranosides using facile and novel biocatalysts of plant origin. *Ind Crops Prod* 74:918–924.
- Yu H-L, Xu J-H, Lu W-Y, Lin G-Q. 2008. Environmentally benign synthesis of natural glycosides using apple seed meal as green and robust biocatalyst. *J Biotechnol* 133:469–477.
- Yu HL, Xu JH, Lu WY. 2007. Identification, purification and characterization of beta-glucosidase from apple seed as a novel catalyst for synthesis of O-glucosides. *Enzyme Microb Technol* 40:354–361.