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RESEARCH ARTICLE

Preparation of salidroside with *n*-butyl β-D-glucoside as the glycone donor via a two-step enzymatic synthesis catalyzed by immobilized β-glucosidase from bitter almonds

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ABSTRACT

β-Glucosidase from bitter almonds was immobilized on epoxy group-functionalized beads for catalyzing salidroside synthesis in a two-step process with *n*-butyl-β-D-glucoside (BG) as the glucosyl donor. The formation of salidroside ((0.59 ± 0.02) M) at a yield of 39.04%±1.25% was accomplished in 8 h by the transglucosylation of immobilized β-glucosidase at pH 8.0 and 50 °C when the ratio of BG to tyrosol was 1:2 (mol/mol). A study on the influence of different glycosyl acceptors demonstrated that the yield of the glucosylation reaction of phenylmethanol and cyclohexanol was higher than that of either phenol or cyclohexanol. This may account for the selectivity of the immobilized enzyme towards the alcoholic hydroxyl group of tyrosol in the salidroside synthesis reaction. A study on the synthesis of BG via the reverse hydrolysis of immobilized β-glucosidase showed that a yield of 78.04%±2.2% BG can be obtained with a product concentration of (0.23 ± 0.015) M.

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Introduction

Salidroside is a glucoside of tyrosol (2-(4-hydroxyphenyl)ethyl-β-D-glucopyranoside), which is a natural product isolated from the roots of a traditional Chinese medicinal plant, *Rhodiola rosea L.* (Zhang et al. 2011). Salidroside possesses biological activities, including the ability to stimulate the nervous system (anti-depression, anti-radiation, and anti-fatigue), reduce hypoxia, and slow the aging process (Shi et al. 2012; Li et al. 2017; Jiang et al. 2018). The large-scale production of salidroside by isolation from wild *Rhodiola spp.* is constrained by the lack of abundant natural sources; this limit motivates research to find alternative approaches for the production of this glycoside. Chemical synthesis of salidroside by the glucosylation of tyrosol has long been studied (Guo et al. 2010; Shi et al. 2011). However, the synthesis of salidroside by the chemical method is considered complicated and expensive, involving selective protection, activation, stereoselective coupling, and deprotection (Guo et al. 2010; Potocká et al. 2015). Bioprocesses using cell suspension culture (Wu et al. 2003) and enzymatic conversion (Tong et al. 2004; Zhang et al. 2005; Akita et al. 2006) have recently been developed for the biosynthesis of salidroside. Enzymatic approaches, on the other hand, can yield anomerically pure glycoside in one step and offer an effective, straightforward, and environmentally friendly alternative to chemical synthesis.

Glucosidases from the seeds of almonds (Akita et al. 2006; Lu et al. 2007; Yu et al. 2007; Yanhong et al. 2012), apples (Tong et al. 2004; Yu et al. 2008; Yang et al. 2012), peaches, prunes, and black plums (Yang et al. 2012) were used to catalyze the glucosylation of tyrosol to salidroside. These reactions were mostly conducted via reversed hydrolysis, i.e. the direct glucosylation of tyrosol by free glucose.

Glucosidase-catalyzed alkyl glycoside synthesis can occur through either reverse hydrolysis or transglycosylation (Van Rantwijk et al. 1999). The former is a thermodynamically controlled synthesis (TCS) reaction and the latter is a kinetically controlled synthesis (KCS) reaction. Thermodynamically controlled reversed

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Supplemental data for this article can be accessed here.

hydrolysis includes a reaction of a monosaccharide with a nucleophile, such as an alcohol, to yield the corresponding glycoside until equilibrium is reached. The TCS of reverse hydrolysis demands an increase in the concentration of glucose or alcohol or a reduction in the water content or activity. A small amount of water is nevertheless usually added, because glycosidases are inactive at low water contents or activity, as well as to increase the solubility of the carbohydrate. A high concentration of the substrate carbohydrate favors a shift in the equilibrium but may lead to the formation of oligomeric side products (Panintrarux et al. 1995) or substrate inhibition at high concentrations of glucose (Wang et al. 2017).

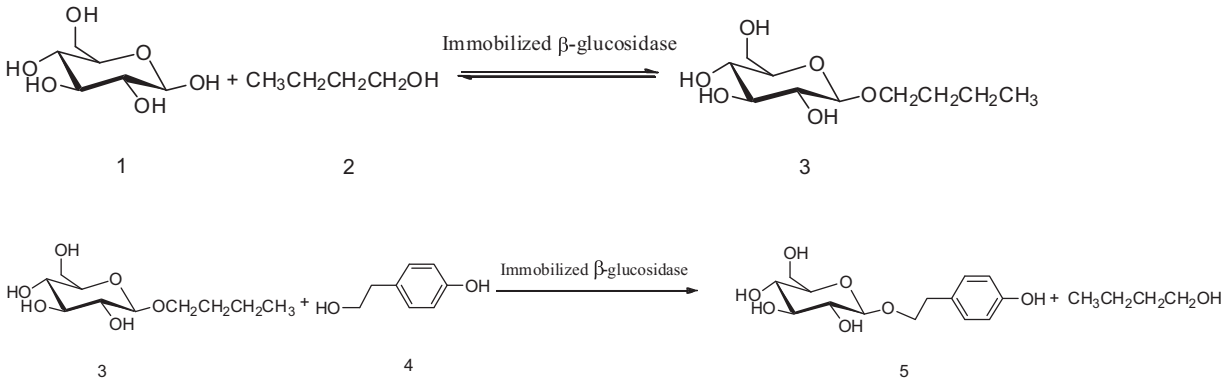
In general, much higher reaction rates and transient yields can be obtained by KCS rather than TCS. During the KCS of transglycosylation, active glycosides are used as glycosyl donors (e.g. p-nitro-phenyl glycoside) and an alcohol acts a nucleophilic acceptor to generate a new glycosidic bond (Crout and Vic 1998). KCS depends more on the properties of the enzyme rather than on the reaction conditions or the thermodynamic equilibrium. The KCS of transglycosylation should be stopped when the rates of the synthesis and dealkylation reaction become equal before thermodynamic control takes over and the product undergoes enzymatic hydrolysis. The choice of a suitable donor is important for glycoside synthesis by transglycosylation. In TCS, a decrease in the water content and activity could result in an increase in the equilibrium yield but this was not the case in KCS, wherein the ratio between transglucosylation and hydrolysis increased with an increase in water content and activity (Andersson and Adlercreutz 2001). An important limitation is the poor water solubility of the glycosyl acceptor which prevents efficient contact with the glycosyl-enzyme intermediate.

In the case of salidroside synthesis, the major drawbacks of glucosidase-catalyzed glucosylation through reverse hydrolysis are as follows. (i) A long reaction time is needed to achieve equilibrium, (ii) the poor solubility of tyrosol in the glucose solution results in a low yield of the glucoside product due to the unfavourable equilibrium position, and (iii) the need for extremely excessive amounts of tyrosol increases the cost of the process (Yu et al. 2008). Increasing the reaction temperature may favor salidroside synthesis, but it might adversely affect the stability of the enzyme.

The synthesis of salidroside by kinetically controlled transglycosylation, on the other hand, has advantages of shorter reaction times and higher yields (Lundemo 2015). Akita et al. found that the yield of glucosidase-catalyzed salidroside synthesis was as high as 36% with 4-nitrophenyl-β-D-glucopyranoside as the glucosyl donor (Akita et al. 1999). Disaccharides were also introduced as glucosyl donors for the synthesis of salidroside. For example, a salidroside yield of 6.7% was achieved with cellobiose as the glucosyl donor by the transglucosylation reaction catalyzed by *Aspergillus niger* glucosidases (Potocká et al. 2015). However, the high cost of 4-nitrophenyl-β-D-glucopyranoside, poor solubility of carbohydrates in organic solvents, and poor solubility of tyrosol in aqueous solutions limit the salidroside synthesis by β-glucosidase catalysis (Potocká et al. 2015).

n-Butyl β-D-glucoside (BG) was used in the past as a glucosyl donor for the glucosylation of long-chain alkyl alcohols in acid-catalyzed transglucosylation reactions (von Rybinski and Hill 1998). BG can dissolve in both organic solvents and water. Thus far, there are no reports of the application of BG as the glucosyl donor in the enzymatic synthesis of salidroside. In the present study, a new two-step enzymatic synthesis procedure is described for salidroside production (Scheme 1). Firstly, BG **3** was synthesized by the glucosylation of *n*-butanol **2** with glucose **1** through reverse hydrolysis, which was catalyzed by β-glucosidase immobilized from bitter almonds. Next, salidroside **5** was prepared by the transglucosylation of the same immobilized enzyme using **3** as the glucosyl donor and tyrosol **4** as the glucosyl acceptor. According to Ismail et al.'s report, the enzymatic synthesis of BG **3** catalyzed by β-glucosidase from almonds results in 41.6 g/L (0.182 M) of BG after optimization by response surface methodology (Ismail et al. 1998). However, at the industrial level, the concentration of BG obtained chemically can exceed 90 g/L (0.394 M) (Matsumura et al. 1990; Urata et al. 1995). Therefore, it is necessary to improve the performance of the enzymatic process to suggest its use at the industrial level, using methods such as enzyme immobilization.

β-Glucosidase (β-D-glucoside glucohydrolase, EC3.2.1.21) is capable of catalyzing the hydrolysis of β-glucosidic linkages in disaccharides, oligosaccharides, and other glycoconjugates (Ismail et al. 1998). It can also be used for glycoside synthesis via reversed hydrolysis or transglycosylation (Van Rantwijk et al. 1999). The β-glucosidase used in this study was extracted and purified from fresh Chinese bitter almonds (*Prunus dulcis var. amara*). Immobilization of the enzyme was carried out using epoxy-functionalized beads (ReliZyme EP113/S).



Scheme 1. Salidroside synthesis with BG as the glucosyl donor via the transglucosylation of the β-glucosidase from bitter almonds immobilized on EP113/S beads. BG is first synthesized from glucose and *n*-butanol by the reverse hydrolysis of the immobilized β-glucosidase.

Material and methods

Material

The bitter almond (*Prunus dulcis var. amara*) sample used for β-glucosidase extraction was a gift from Zhangye Chinese Medicine Technology Co., Ltd., Gangsu, China. Tyrosol **4**, salidroside **5**, 4-nitrophenyl β-D-glucopyranoside (pNPG) **6**, *p*-nitrophenol (pNP) **7**, *tert*-butyl alcohol **8**, phenol **9**, hydroquinone **10**, phenylmethanol **11**, cyclohexanol **12**, cyclohexylmethanol **13**, 2-isopropyl-5-methylcyclohexanol **14**, benzyl β-D-glucopyranoside **15**, 4-hydroxyphenyl β-D-glucopyranoside **16**, and phenoxyl β-D-glucopyranoside **17** were purchased from Sigma Aldrich, Shanghai Trading Co. Ltd. (Shanghai, China). Neomenthyl β-D-glucopyranoside **18** was purchased from Extrasynthese (Genay, France). Cyclohexylmethyl β-D-glucopyranoside **19** and cyclohexyl β-D-glucopyranoside **20** were procured from Carbosynth China Ltd. (Suzhou, China). Silica gel (Davisil grade 633, 200–425 mesh) for column purification was purchased from Aldrich Fisher Scientific. Epoxy group-functionalized resin beads, ReliZyme EP113/S, were purchased from Re Sindion S.R.L. (Binasco, Italy). The average pore size of the beads was 20–50 nm and their diameter was in the range of 100–300 μm. All other agents used were of analytical grade and supplied by Shanghai Sinopharm Chemical Reagent Co., Ltd.

β-glucosidase preparation

β-Glucosidase (2.16 U/mg protein, 68 kDa MW) was extracted and purified from fresh bitter almonds (*Prunus dulcis var. amara*) according to a previously described method with some modifications (Wang et al. 2017). The enzyme extract was purified by ultrafiltration in a stirred cell (Model 8050, Amicon Div., Millipore Corp.) equipped with flat-sheet membranes.

Table 1. Extraction and purification of β-glucosidase from bitter almonds.

	Crude extract	100 kDa MWCO ultrafiltration	50 kDa MWCO ultrafiltration
Protein content (mg/ml)	266.42	74.82	57.46
Enzyme activity (U/mg protein)	0.58	1.74	2.16

The enzyme extract was placed in the cell and filtered successively with membranes with molecular weight cut-off (MWCO) values of 50 kDa and 100 kDa (Millipore Corp., Bedford, MA). The filtrates containing substances of molecular weights in the range of 50 kDa to 100 kDa were collected. The collected filtrate was freeze-dried to obtain crude β-glucosidase. The results of the purification procedure are listed in Table 1.

Later, β-glucosidase immobilization was carried out with ReliZyme EP113/S beads as the support. The resin beads were washed twice with a solution (95% ethanol) ten times their weight for 20 min and twice with deionized water for 30 min. After washing, 150 mg of the beads were incubated with 28 mg of crude β-glucosidase in 12 mL of a 50 mM sodium phosphate buffer (pH 8.0) of 1 mmol cellobiose for 6 h at room temperature on a shaker at 150 rpm. At the end of the incubation period, the immobilized enzyme was collected by centrifugation and washed thrice with the same phosphate buffer to remove any residual "free" enzyme from the beads. The amount of enzyme immobilized was estimated by determining the amount of the enzyme in the solution and wash buffers. From the obtained values, the loading of β-glucosidase was determined to be 158 mg/g beads. The specific activity of the immobilized enzyme was 1.82 U/mg protein. The total activity of the β-glucosidase-immobilising beads was 287.6 U/g. The immobilized

β-glucosidase thus obtained was used in a two-step enzymatic reaction consisting of a reverse hydrolysis for BG synthesis and a transglucosylation reaction for salidroside synthesis.

A bicinchoninic acid (BCA) assay was used to quantify the immobilized protein by measuring the protein concentration in solution using bovine serum albumin as the standard (Smith et al. 1985). The activity of β-glucosidase was determined by measuring the release of **7** from glucoside **6** hydrolyzed by free β-glucosidase and the immobilized derivative (Wang et al. 2017). The amount of enzyme used for the assay was 0.2 mL for the free enzyme and 20 mg for the immobilized enzyme. One unit of β-glucosidase activity was defined as the amount of enzyme that released 1.0 μmol **7**/min at 50 °C.

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

The effect of temperature on the activity of β-glucosidase immobilized on EP113/S beads was determined at pH 7.0 in the temperature range of 20 to 80 °C. The effect of pH on the activity of immobilized β-glucosidase was determined at 50 °C in 50 mM citric acid-citrate buffer (pH 4.0–6.0), 50 mM sodium phosphate buffer (pH 6.0–8.0), and 50 mM glycine-sodium hydroxide buffer (pH 9.0). The relative activity (%) was calculated by comparing the activity of every sample against the maximum activity (defined as 100%).

Evaluation of storage stability and thermal stability of immobilized β-glucosidase

The storage stabilities of free and immobilized β-glucosidase were measured by storing the enzymes at 4 °C for 6 days and determining the residual activity at every 24 h.

The thermal stabilities of free and immobilized β-glucosidase were evaluated by incubating the enzymes at 30–100 °C for 12 h in a 50 mM sodium phosphate buffer (pH 7.0). The enzymes were then cooled in an ice bath and their residual activities were measured. The initial activity of each enzyme was defined as 100%.

n-Butyl-β-D-glucoside 3 syntheses by immobilized β-glucosidase-catalyzed reverse hydrolysis

To a 2-mL mixture of **2** and the sodium phosphate buffer (50 mM), 0.6 mM **1** and 5 U of immobilized β-glucosidase were added and allowed to react at 50 °C and 600 rpm. The reaction could be easily monitored by reverse phase high-performance liquid chromatography (HPLC) (Figure 1S) and was terminated when the formation of the desired product reached the maximum value. At the end of the reaction period, the reaction mixture was filtered through a 0.22-μm membrane to remove the immobilized enzyme. The amount of glucoside **3** in the filtrate was determined by HPLC.

The yield of **3** was calculated according to the following equation:

$$\text{BG yield } \% = \frac{m_1}{m_2} \times 100\% \quad (1)$$

where m_1 is the amount of **3** produced under the used reaction conditions (mmol) and m_2 is the amount of glucose used for the synthesis of **3** (mmol).

The filtrate was dried under vacuum at 80 °C. The dried mixture was later extracted with acetone and dried in vacuum. The extract was purified on a silica gel column (1.0 cm ×20.0 cm) under the following conditions – 2 g of the extract in 5 mL acetone was placed gently in a silica-gel column and eluted with a mixture of ethyl acetate/methanol (6/1 v/v). The amount of **3** in the eluent was detected by HPLC. The eluents containing **3** were pooled and dried using a rotary evaporator at 40 °C under vacuum to remove the solvents. The nuclear magnetic resonance (NMR) (^{1}H and ^{13}C NMR) data of **3** was found to be identical with that reported previously (Ismail et al. 1998; Makropoulou et al. 1998).

The data shown in the figures and tables represent average values and standard errors of experiments repeated three times.

Salidroside 5 synthesis by transglucosylation of the immobilized β-glucosidase

In a mixture of 1.76 mL of **8** and 0.04 mL of 50 mM sodium phosphate buffer (pH 7.0), 6 mmol **4** and 3 mmol **3** were dissolved. Upon the addition of 5 U of immobilized β-glucosidase, the reaction was initiated and it was conducted on a rotary shaker at 50 °C and 600 rpm. The reaction was monitored by reverse phase HPLC (Figure 2S) and terminated when the formation of the desired product reached a maximum value. At the end of the reaction, the mixture was filtered through a 0.22-μm membrane to remove the immobilized enzyme. The content of **5** in the filtrate was detected by HPLC.

The yield of **5** was calculated according to the following equation:

$$\text{Salidroside yield } \% = \frac{m_3}{m_4} \times 100\% \quad (2)$$

where m_3 is the amount of **5** produced under the used reaction conditions (mmol) and m_4 is the amount of **3** used for the synthesis of **5** (mmol).

The filtrate was evaporated at 80 °C under vacuum and the dried mixture was extracted with acetone and dried in vacuum. The extract was purified by a silica gel column under the following conditions – 2 g of the extract in 5 mL of acetone was added to the silica gel column (1.0 cm ×20.0 cm) gently and eluted with an ethyl acetate/methanol (4/1 v/v) mixture. The amount of **5** in the eluent was detected by HPLC. The eluents containing **5** were pooled and dried on a rotary evaporator at 40 °C in vacuum to remove the solvents. The NMR (^{1}H and ^{13}C NMR) data of **5** were identical with those reported previously (Delépée et al. 2007) (Akita et al. 2006).

Determination of the kinetic parameters of the synthesis reaction of 5

The kinetics of glucose (0.3 M) addition during the synthesis of **5** by immobilized β-glucosidase are described using a reduced Haldane equation. 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.

Reusability of the immobilized enzyme

The reusability of immobilized β-glucosidase for the synthesis of **5** was examined by measuring the residual activities of the immobilized enzymes after repeated operations. In a mixture of 1.76 mL of **8** and 0.04 mL of 50 mM sodium phosphate buffer (pH 7.0), 6 mmol **4** and 3 mmol **3** were dissolved. Upon the addition of 5 U of immobilized β-glucosidase, the reaction was initiated and it was allowed to continue on a rotary shaker at 50 °C and 600 rpm for 8 h. After each run, the immobilized enzyme was separated by filtration, washed with a phosphate buffer of pH 7.0, and reintroduced into the fresh reaction medium.

General procedure for the glucosylation of other aglycones

The general procedure for the glucosylation of other glucosyl acceptor-like compounds, **7**, **9**, **10**, **11**, **12**, **13**, and **14**, was similar to the procedure used for the synthesis of **5**. To a mixture of 1.76 mL of **8** and 0.04 mL of 50 mM sodium phosphate buffer (pH 7.0), 6 mmol of the selected glucosyl acceptor and 3 mmol **3** were added. The reaction was initiated by adding 5 U of immobilized β-glucosidase; it was carried out on a rotary shaker at 50 °C and 600 rpm. The product yield was determined by HPLC. At the end of the reaction, the mixture was filtered through a 0.22-μm membrane to remove any immobilized enzyme. The filtrate was evaporated under vacuum and the residue was extracted with acetone and purified on a silica gel column (eluted with ethyl acetate/MeOH: 4/1–13/1). The structures of the glucoside products were confirmed by NMR.

Characterization of the compounds

HPLC analysis (Model 1525, Waters, U.S.A) was carried out on an Aminex HPX-87H Column (Bio-Rad, φ300 mm ×7.8 mm, 9 μm); the instrument was equipped with a Model 2420 Evaporative Light Scattering (ELSD) detector. The flow rate of the eluent and the column temperature were 0.6 mL/min and 40 °C, respectively. Gradient elution with acetonitrile/water (25/75 (v/v)) from 0 to 5 min, 75/25 (v/v) from 5 to15 min, 100% acetonitrile from 15 to 22 min, and 25/75 (v/v) from 22 to 25 min was performed. Glucoside peaks were identified by comparison with the retention time of authentic standards. Yields were calculated from the ratio of the product concentration to the initial concentration of **3**.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance HD III 400 MHz spectrometer (Bruker, Wormer, Netherlands) using 10–20 mg of the samples in 0.5 mL MeOD with Me_4Si as the internal reference. Relative molecular masses were determined on a Micromass Quattro Micro API mass spectrometer (MS) (Waters Corp., Milford, MA) coupled to a Shimadzu LC-10ADvp HPLC system (Shimadzu, Kyoto, Japan). The data obtained in the positive electrospray ionization (ESI) mode was analyzed using MassLynx V 4.1.

3: ^{1}H NMR (400 MHz, MeOD) δ (ppm) 4.28 (d, *J* = 7.8 Hz, 1*H*), 3.90 (m, 2*H*), 3.69 (dd, *J* = 11.9, 5.2 Hz, 1*H*), 3.56 (dd, *J* = 9.6, 6.7 Hz, 1*H*), 3.30–3.36 (m, 3*H*), 3.21 (t, *J* = 8.0 Hz, 1*H*), 1.68–1.55 (m, 2*H*), 1.48–1.34 (m, 2*H*), 0.95 (m, 3*H*). ^{13}C NMR δ (ppm) 104.3 (C_1), 78.1, 77.9, 75.1, 71.62, 70.56, 62.72, 32.88, 23.94, and 15.45. ESI-MS (*m/z*): 259.1 (M+Na^+). **5**: ^{1}H NMR (400 MHz, MeOD) δ (ppm) 7.08 (d, *J* = 8.4 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 2H), 4.31 (d, *J* = 7.8 Hz, 1H), 4.05 (m, 1H), 3.89 (m, 1H), 3.70 (m, 2H), 3.37 (t, *J* = 8.8 Hz, 1H), 3.30 (d, *J* = 7.0 Hz, 2H), 3.20 (t, 1H), 2.87 (t, 2H). ^{13}C NMR δ (ppm) 156.8, 130.9, 130.9, 130.7, 116.1, 116.1, 104.4,

78.1, 77.9, 75.1, 72.1, 71.6, 62.7, and 36.4. ESI-MS (*m/z*): 323.1 ($M+Na^+$). **6**: ^{1}H NMR (400 MHz, MeOD) δ (ppm) 3.13–3.22 (dd, 3*H*), 3.79–3.84 (d, 2*H*), 5.85 (d, J = 2.7 Hz, 1*H*), 7.37 (ddd, J = 8.0, 1.2, 0.4 Hz, 2*H*), 8.16 (ddd, J = 8.0, 2.1, 0.4 Hz, 2*H*). ^{13}C NMR δ (ppm) 101.2(C_1), 157.7, 144.1, 71.2, 117.4, 77.6, 77.0, 74.2, 125.7, and 62.1. ESI-MS (*m/z*): 324.1 ($M+Na^+$). **15**: ^{1}H NMR (400 MHz, MeOD) δ (ppm) 3.17 (dd, 3*H*), 3.53 (dt, J = 10.3, 6.6 Hz, 1*H*), 3.79–3.83 (d, 2*H*), 4.67–4.68 (s, 2*H*), 7.28–7.47 (m, 5*H*). ^{13}C NMR δ (ppm) 103.5(C_1), 136.5, 128.9, 128.5, 77, 62.1, 77.6, 127.9, 73.9, 70.0, and 71.2. ESI-MS (*m/z*): 293.1 ($M+Na^+$). **16**: ^{1}H NMR (400 MHz, MeOD) δ (ppm) 3.13–3.21 (dd, 3*H*), 3.53 (dt, J = 10.3Hz, 1*H*), 3.79–3.84 (d, 2*H*), 5.08 (d, J = 2.7 Hz, 1*H*), 6.81–6.92 (ddd, 4*H*). ^{13}C NMR δ (ppm) 101.234(C_1), 151.937, 77.0, 150.3, 115.44, 77.6, 116.1, 62.1, 71.2, and 74.2. ESI-MS (*m/z*): 295.1 ($M+Na^+$). **19**: ^{1}H NMR(400 MHz, MeOD) δ(ppm) 1.21–1.62 (m, 10*H*), 1.68 (ttt, J = 10.3, 5.9, 2.8 Hz, 1*H*), 3.13–3.21 (dd, 3*H*), 3.53 (dt, J = 10.3, 6.6 Hz, 1*H*), 3.71–3.75 (d, 2*H*), 3.79–3.83 (d, 2*H*), 4.47 (d, J = 2.7 Hz, 1*H*). ^{13}C NMR δ (ppm) 104.7(C_1), 38.9, 77.6, 77.0, 73.9, 71.2, 70.0, 62.1, 26.3, 28.3, and 25.3. **20**: ^{1}H NMR (400 MHz, MeOD) δ (ppm) 1.37 (dquint, J = 12.7, 2.8 Hz, 1*H*), 1.35–1.76 (m, 9*H*), 3.13–3.21 (dd, 3*H*), 3.47–3.74 (m, 2*H*), 3.79–3.83 (d, 2*H*), 4.48 (d, J = 2.7 Hz, 1*H*). ^{13}C NMR δ (ppm) 102.8 (C_1), 77.6, 77, 74.1, 72.5, 71.2, 62.1, 31.6, 25.3, and 23.4. ESI-MS (*m/z*): 285.1 ($M+Na^+$).

Evaluation of the influence of various non-aqueous media on the synthesis of 5

The influence of various non-aqueous media on the synthesis of **5**, catalyzed using immobilized β-glucosidase, was evaluated using the following procedure. To a medium containing a mixture of 50 mM sodium phosphate buffer (pH 8.0) and an organic solvent, 6 mmol **4** and 3 mmol **3** were added. The reaction was initiated by the addition of 5 U of immobilized β-glucosidase and carried out on a rotary shaker at 50 °C and 600 rpm. At the end of the reaction period, the mixture was filtered through a 0.22-μm membrane to remove the immobilized enzyme. The filtrate was evaporated at 80 °C in vacuum. **5** in the dried mixture was extracted using acetone and purified on a silica gel column.

Evaluation of stability of the immobilized β-glucosidase in the presence of various glucosyl acceptors

The stabilities of the immobilized enzymes in *t*-butanol solutions of the glucosyl acceptors, **7**, **9**, **10**, **11**, **12**, **13**, and **14**, were examined. To a mixture of 1.76 mL of *t*-butanol and 0.04 mL of 50 mM sodium phosphate buffer (pH 7.0), 6 mmol of the glucosyl acceptor and 5 U of immobilized β-glucosidase were added. Incubation was carried out on a rotary shaker at 50 °C and 600 rpm. In the control experiment, 50 μL of 50 mM sodium phosphate buffer (pH 7.0) was used instead of a glucosyl acceptor. After 24 h of incubation, the enzyme was separated and its activity was determined. The residual β-glucosidase activity was expressed as % residual activity compared to the initial activity of the enzyme.

Results and discussion

Influence of EP113/S immobilization on the activity and stability of β-glucosidase from bitter almonds

Supports containing epoxy groups can generate multipoint covalent attachment with different nucleophiles located on the surfaces of enzyme molecules (e.g. amino, thiol, and hydroxyl groups) (Mateo et al. 2007a). The multipoint covalent linkages between, for example, β-glucosidase and EP113/S beads, involve most of the subunits to increase the overall rigidity of the protein, thus enhancing the stability of the enzyme (Fernandez-Lafuente 2009). Immobilization of enzymes is often accompanied by a partial loss of activity, which can often be minimized by loading the active sites with a reactant or a reactant mimic to prevent unfavorable modification of the active sites (Gupta, 1993). All β-glucosidases require a β-glycoside (i.e. glucoside and to a much lesser extent, fucoside and galactoside) as the substrate (Esen 1993). In this study, in order to maintain the active conformation of bitter almond β-glucosidase during immobilization on EP113/S beads, cellobiose, a disaccharide consisting of two β-glucose molecules linked by a $\beta(1 \rightarrow 4)$ bond, was added to the enzyme solution. This incubation resulted in the blocking of the substrate-binding active sites of the enzyme with cellobiose, resulting in the protection of the active sites from chemical modification by the epoxy groups on EP113/S beads (Roig et al. 1986; Mateo et al. 2007b); further, the active conformation was fixed by multi-subunit immobilization of enzymes. Upon the application of this strategy, the specific activity of immobilized β-glucosidase was found to be 1.82 U/mg protein, which is 84.25% of the specific activity of the free enzyme but higher than that obtained in the case without cellobiose addition (1.23 U/mg protein). A similar strategy was employed for the immobilization of *A. oryzae*

β-galactosidase (Woudenberg-van Oosterom et al. 1998). The activity of *A. oryzae* β-galactosidase immobilized in the presence of 0.1 M lactose was higher than that obtained when there was no lactose addition.

The effect of immobilization with EP113/S beads as the support on the properties of β-glucosidase from bitter almonds is demonstrated in Figure 1. Changes in the activity of free β-glucosidase and the immobilized derivative were examined at pH 7.0 over a temperature range of 20 °C to 80 °C (Figure 1(A)). First of all, the activities of both the free and immobilized enzymes increased with an increase in the temperature up to 50 °C. When the temperature increased from 50 °C to 60 °C, the activity of the free enzyme decreased sharply to about 80% of its initial activity, while the immobilized enzyme retained more than 90% of its initial activity. When the temperature was increased beyond 80 °C, the residual relative activity of free β-glucosidase and the immobilized derivative decreased to 20%±2.3% and 50.9%±3.2%, respectively. Multipoint covalent attachment of β-glucosidase onto the EP113/S beads increased the thermal stability of the enzyme molecules. A similar result was observed in a study on the immobilization of β-glucosidase on silicon oxide nanoparticles (Singh et al. 2011).

The activation energies of the free β-glucosidase and immobilized derivative-mediated reactions were calculated using the Arrhenius equation in the region where temperature-dependent denaturation did not occur (Wang et al. 2014). The activation energy was calculated to be 13.25 kJ/mol for the free enzyme and 9.35 kJ/mol for the immobilized derivative. The slightly reduced activation energy of the reaction catalyzed by immobilized β-glucosidase indicates that the more favorable active conformations of the enzyme molecules were probably obtained after immobilization.

The protonation status of catalytic amino acids (glutamate or aspartate) at the active sites of β-glucosidase determines its activity, which is further influenced by pH (Jonathan and Arnold 1981). The pH-activity profiles of free and immobilized β-glucosidases at 50 °C are shown in Figure 1(B). The relative activity of both the free and immobilized enzymes increased

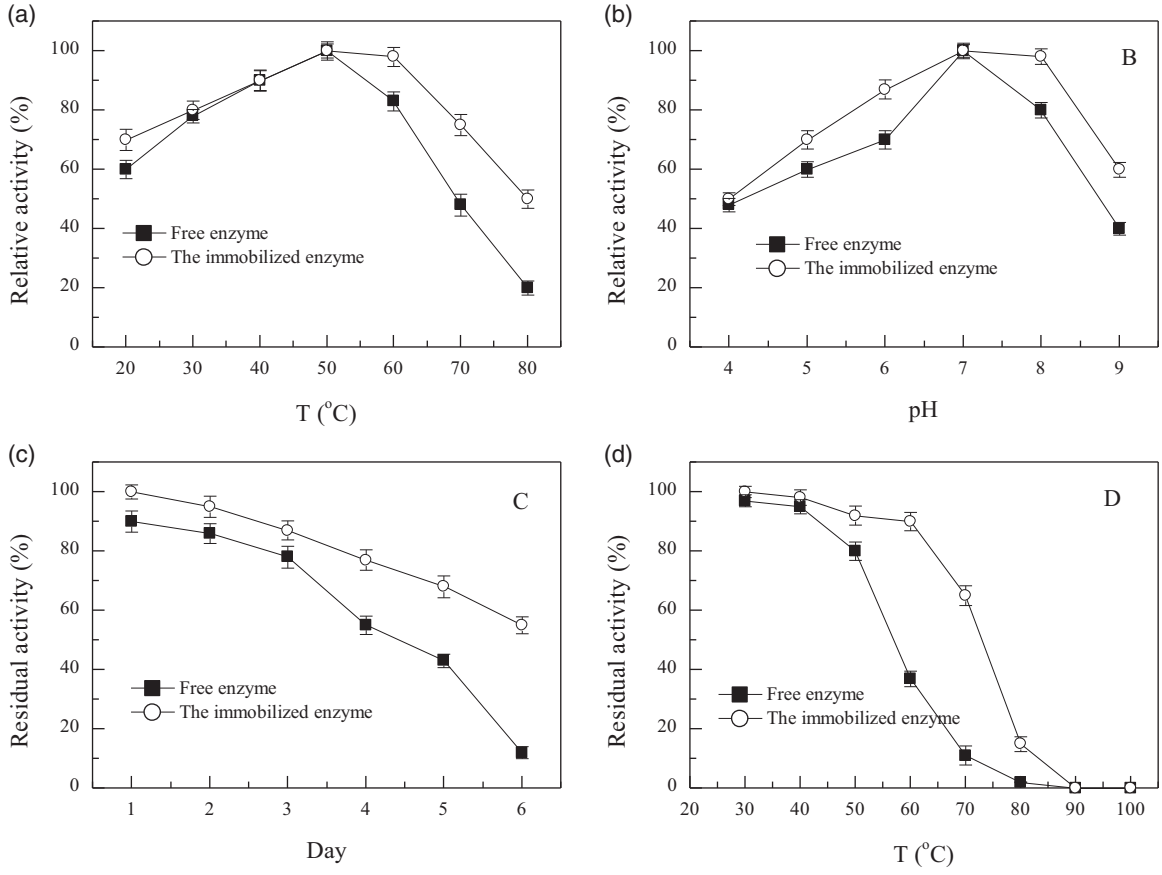


Figure 1. The influence of immobilization with EP113/S beads on the activity of β-glucosidase from bitter almonds at various pH and temperature, and the investigations of influences of the immobilization on the thermal stability and storage stability of the enzyme.

with an increase in the pH and reached the maximum at pH 7.0. In the pH range of 7.0–9.0, the activity of the immobilized enzyme was higher than that of the free enzyme. This is because multipoint covalent attachment of enzyme molecules to the support increases the rigidity of the enzyme conformation, which in turn increases its stability with respect to pH.

During a study on storage stability, we found that the free enzyme lost more than 90% of its initial activity in 6 days. In contrast, the immobilized enzyme retained ≈ 54.2% of the initial activity (Figure 1(C)). The thermal stabilities of free β-glucosidase and the immobilized derivative were also investigated and the results are shown in Figure 1(D). The immobilized enzyme retained about 60% of its initial activity, while free *β*-glucosidase lost almost 90% of its activity after incubation at 70 °C for 12 h. This may be because the epoxy groups on the EP113/S beads stabilized the three-dimensional structure of the enzyme at elevated temperatures (Mateo et al. 2002). Other reports on the application of epoxy-activated supports for the immobilization of enzymes found a significant improvement in the thermal stability of *β*-glucosidase from *Issatchenkia terricola* and *Aspergillus niger* (Tu et al. 2006; González-Pombo et al. 2011).

Synthesis of salidroside 5 via the transglucosylation of the immobilized β-glucosidase with BG 3 as the glucosyl donor

Although the synthesis of salidroside **5** via the reverse hydrolysis of *β*-glucosidase is elegant in its simplicity (Chen et al. 2012; Yanhong et al. 2012), the poor miscibility of tyrosol in the glucose solution leads to a low yield. BG **3** has been used as the glucosyl donor for the glucosylation of long-chain alkyl alcohols in chemical processes. In this study, the possibility of using **3** as the glucosyl donor in the synthesis of **5** through transglucosylation of the immobilized *β*-glucosidase was investigated and the results are presented in Figure 2.

A yield of 39.04%±1.25% for **5** was obtained using a *t*-butanol solution containing 2% (v/v) water. Further reduction or increase in the water content of the *t*-butanol solution resulted in reduced yields. Water content is a critical experimental parameter impacting the activity of *β*-glucosidase and the reaction equilibrium of glucosylation (Tong et al. 2005). This is because (i) enzymatic activity is inhibited due to a lack of an adequate water content for maintaining the active conformation of the enzyme and (ii) the hydrolysis

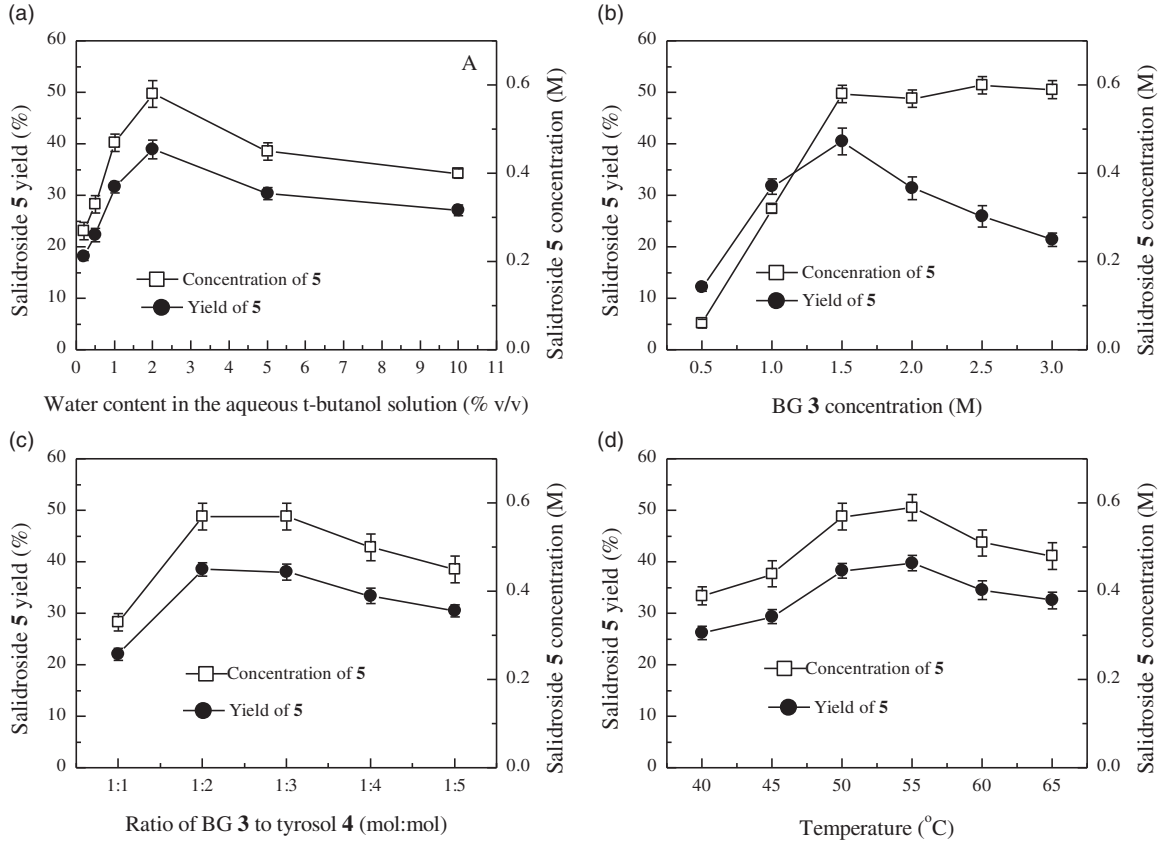


Figure 2. Influence of various water content in *t*-butyl alcohol solution (A), different concentrations of **3** (B), different ratio of **3** to **4** (C) and different temperature (D) on the yield of **5**. (The reactions were carried out at pH 7.0 and 50 °C. The immobilized β-glucosidase used was 2.5 U/mL of medium).

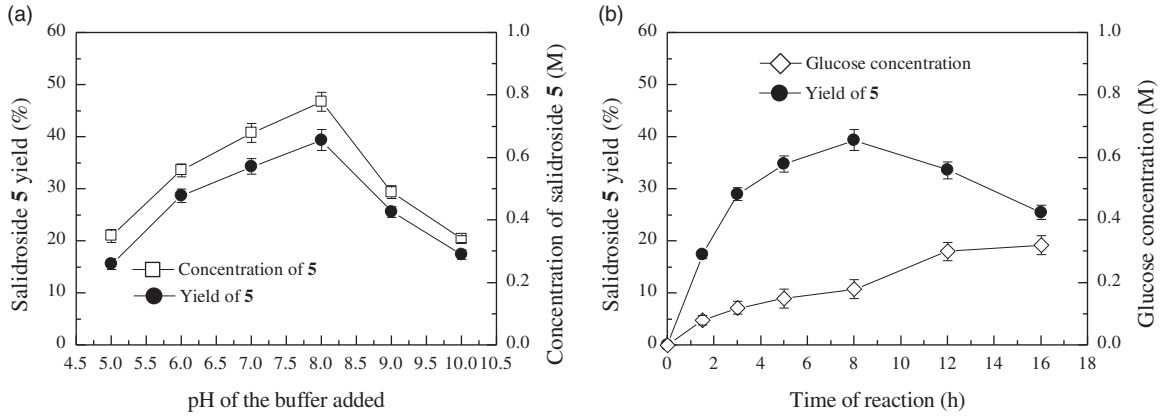


Figure 3. Influences of pH of the buffer added (A) and reaction time (B) on the yield and the product concentration of **5**. (The reactions were carried out at 50 °C in the medium containing 2% (v/v) buffer, 1.5 M of **3** when the ratio of **3** to **4** was 1:2. The immobilized β-glucosidase used was 2.5 U/mL of medium).

activity of the enzyme increases in the presence of excess water, which reduces the yield of **5**. Multipoint immobilization of β-glucosidase on EP113/S beads via the epoxy groups increased the rigidity of the three-dimensional structure of an enzyme, thus helping to maintain the active conformation of the enzyme (Yu et al. 2008; Rodrigues et al. 2013; Wang et al. 2017).

The effects of various initial concentrations of **3** and different molar ratios of **3** and **4** on the synthesis of **5** were also investigated (Figure 2(B,C)). The yield of **5** increased and reached the maximal value when the concentration of **3** was 1.5 M. According to Figure 2(C), the maximum yield of **5** was achieved when the ratio of **3** to **4** was 1:2 (mol: mol). The yield of **5** declined gradually as the ratio increased to more than 1:3. This may be due to an increase in the medium viscosity caused by elevated concentrations of **4** in the reaction medium; the increased viscosity results in an increased resistance to substrate diffusion to the active sites of the enzyme (Wang et al. 2017). Temperature is an important factor in enzyme-catalyzed reactions as it affects enzyme activity, stability, and the thermodynamic parameters of the reaction. The influence of different temperatures on the synthesis of **5** via the transglucosylation of immobilized β-glucosidase with **3** as the glucosyl donor is illustrated in Figure 2(D). In the temperature range of 40 °C to 55 °C, the yield increased with an increase in the temperature. When the reaction temperature was further increased to 60 °C or more, the yield dropped markedly. This may be due to the reduced enzyme activity at higher temperatures.

The pH value of the water added to the reaction medium can alter the state of charge of the active sites of the enzyme (Klibanov 1997), resulting in a change in the yield of **5**. The yield and product concentration of **5** reached their maximum values at pH

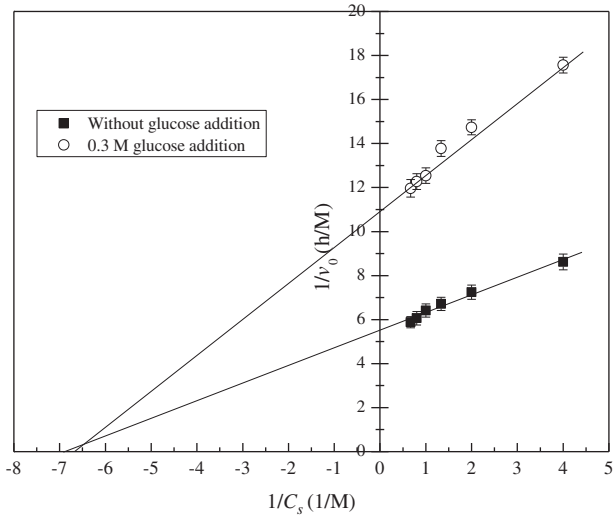


Figure 4. Influence of addition of 0.3 M **1** on the initial reaction velocity (v_0) and Dixon plots of the synthesis of **5** by the immobilized β-glucosidase.

8.0, indicating that a weakly alkaline environment is suitable for the transglucosylation reaction with immobilized β-glucosidase (Figure 3(A)). According to the catalysis mechanism for the transglucosylation reaction of β-glucosidase (Koshland 1953; Yang et al. 2017), weakly alkaline conditions may favor the deprotonation of active sites attacking the glucosidic bonds and forming a glucosyl-enzyme intermediate, which is attacked by nucleophile **4** to generate **5**. According to Figure 3(B), the yield of **5** reached the maximum value when the reaction time was 8 h. The increase in the concentration of glucose **1** in the reaction medium suggested the occurrence of hydrolysis of **3** and/or **5** during the reaction. Finally, the glucosylation reaction catalyzed by immobilized β-glucosidase resulted in **5** with a yield of 39.4%±2.0% and the corresponding product concentration was (0.59 ± 0.02) M.

Kinetic analysis (Figure 4) was carried out to understand the influence of the presence of glucose **1** (0.3 M) on the kinetic parameters of the synthesis of **5**, which was catalyzed by immobilized β-glucosidase at 50 °C and pH 8.0 with **3** as the glucosyl donor. The Dixon plots of the initial reaction velocity (v_0) against the concentration of **3** clearly indicated that v_0 was inhibited when 0.3 M **1** was added at the beginning of the reaction. This inhibition phenomenon was predominantly non-competitive (Stambaugh and Post 1966). According to the Haldane equation, the predicted maximal value of v_0 for the enzyme was 0.18 M/h without **1** addition and 0.09 M/h when 0.3 M **1** was added. On the basis of the Dixon plots (Figure 4), the value of the inhibition dissociation constant (K_{SI}) was calculated as 0.0658 M, implying that glucose inhibition during the synthesis of **5** occurred at a low level (content) of **1**. Non-competitive inhibition of glucose on the enzymatic activity was also reported elsewhere (Reshmi and Sugunan 2013). The effect of a non-competitive inhibitor cannot be reversed by increasing the substrate concentration (Najafpour 2007). Hence, screening for β-glucosidases with a higher tolerance for glucose inhibition may be undertaken to improve the product yield of **5**.

Synthesis of 5 in various non-aqueous media and reusability of the immobilized enzyme

The use of a non-aqueous reaction medium with hydrolases, such as glucosidases, often results in a shift in the thermodynamic equilibrium of enzymatic activity to favor synthesis over hydrolysis (Wang et al. 2016). *t*-Butanol, 1,4-dioxane, dimethyl sulphoxide (DMSO), acetone, and acetonitrile have been reported to be widely used in non-aqueous systems for enzymatic catalysis (Karagulyan et al. 2008). In this study, these solvents were tested in the synthesis of **5** catalyzed by immobilized β-glucosidase with **3** as the glucosyl donor. Among these solvents, the highest yield of **5** was obtained in the *t*-butanol aqueous solution (2% water v/v) (Figure 5). The product yield was almost zero when the water content was less than 1% (v/v) in aqueous acetone and aqueous acetonitrile. According to Tong et al. (Tong et al. 2004), 1,4-dioxane is an effective co-solvent for glucosylation reactions catalyzed by β-glucosidase from apple seed meal. We observed that the yield of **5** obtained with aqueous 1,4-dioxane was comparable to that obtained with aqueous *t*-butanol when the water content was 5% (v/v) (Figure 5). However, when the water content was reduced, the product yield obtained with aqueous *t*-butanol was higher than that obtained with aqueous 1,4-dioxane. This may be due to the difference in the polarities of the organic solvents, which in turn affected the activity of the enzyme. Log *p* is a quantitative measure of the solvent polarity. According to Laane et al. (Laane et al. 1987), the log p-value of *t*-butanol is the highest among the solvents tested in this study, which may account for the higher yield of **5** in aqueous *t*-butanol under the used experimental conditions.

The reuse of enzymes permits simplification of the reactor design and a better control over the reaction (Kharrat et al. 2011). On the basis of the results shown in Figure 6, β-glucosidase extracted from bitter almonds and immobilized on EP113/S beads lost about 65% of its activity after four rounds of recycling, from 39.6%±1.6% (first run) to 10.2%±1.2% (fourth run). Enzyme deactivation is possibly the main reason for the reduction in the enzymatic activity during the recycling operation. The leakage of the enzyme from

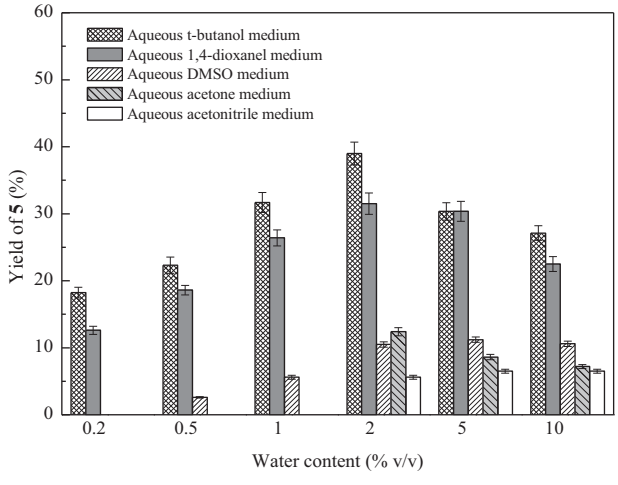


Figure 5. Synthesis of **5** catalyzed by the immobilized β-glucosidase with glucoside **3** as the glucosyl donor in various aqueous organic solvents.

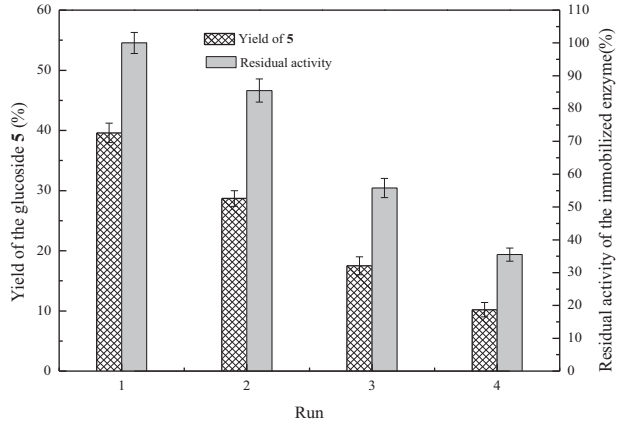


Figure 6. Reusability of the immobilized β-glucosidase for the synthesis of **5**.

the support may be another cause behind the reduced activity. Meanwhile, the enzyme immobilized by covalent coupling between the epoxy groups of EP113/S beads and β-glucosidase can avoid such leakage during the recycling operation. From these results, it can be inferred that there the reusability of immobilized β-glucosidase can be improved by choosing suitable carriers and protein engineering.

Selectivity of the immobilized β-glucosidase towards phenolic hydroxyls and alcoholic hydroxyls

Enzymatic formation of glycosidic bonds via the transglucosylation reaction of β-glucosidase is thought to be mechanistically similar to the acid-catalyzed formation of glycosides (Drueckhammer et al. 1991). It is generally accepted that stereoelectronic effects play an important role in the glycosylation processes (Deslongchamps 1993) and the attack of nucleophile can suffer from steric hindrance at the active site of the enzyme.

In the molecular structure of the glucosyl acceptor **4**, there are two types of alcoholic nucleophiles, a primary alcoholic hydroxyl group and a phenolic hydroxyl group. Both these groups can potentially attack the anomeric carbon of the glucosyl-enzyme intermediate. In order to understand the selective glucosylation of the primary alcoholic hydroxyl group of **4** during the transglucosylation reaction catalyzed by immobilized β-glucosidase from bitter almonds, several glucosyl acceptors, including **7**, **9**, **10**, **11**, **12**, **13**, and **14** were tested for transglucosylation with **3** as the glycosyl donor. The two acceptors were **11** and **13**, which can produce high yields of glucoside products, **15** (63.6%±2.3%) and **19** (78.6%±2.5%), among the alcohols and phenols tested (Table 2). When the hydroxyl group is directly connected an alkyl ring or a benzene ring, the glucoside yield decreased significantly; for instance, the yields of glucoside **17** and **20** were zero and 10.2%±1.5%, respectively. Generally, glycosidases cannot access secondary or tertiary alcohols as aglycone acceptors (Drueckhammer et al. 1991; Rather and Mishra 2013). In this investigation, it was found that the primary hydroxyl group of **4** can be glycosylated selectively, whereas the phenolic hydroxyl group of **4** cannot be glycosylated. It was also found that the yield of glucoside **20** obtained via the glucosylation of **12** was higher than the yields of **6**, **16**, and **17**, which were obtained by the glucosylation of **7**, **10**, and **9**, respectively (Table 2). In contrast to the rigid structure of the benzene ring of phenols, the higher flexibility of the cyclohexane ring of **12** may allow partial rotations (twists) about C-C single bonds to meet the requirement of active sites on the enzyme (Dougherty 1996). Similarly, due to sterical hindrance imposed by the isopropyl group, the hydroxyl group of **14** cannot be glycosylated by immobilized β-glucosidase with **3** as the glycosyl donor. An investigation on the stability of the immobilized β-glucosidase in the presence of the glucosyl acceptors was also carried out. It was found that the residual activity of the immobilized β-glucosidase was approximately equal to the residual activity of the immobilized enzyme in the absence of an acceptor (control experiment) (Figure 7), after incubating in *t*-butanol solutions of **7**, **9**, **10**, **11**, **12**, **13**, and **14** for 24 h at 50 °C. Hence, the difference in the yields obtained from the enzymatic glycosylation of various glucosyl acceptors can be ascribed to the selectivity of the enzyme with respect to different types of hydroxyl groups.

Table 2. β-glucosides of **7**, **9**, **10**, **11**, **12**, **13**, and **14** prepared by the transglucosylation of β-glucosidase.

Glucosyl acceptor	Target molecule	Reaction time (h)	Yield (%)
4	**5**	8	39.04 ± 1.25
7	**6**	6	1.8 ± 0.22
9	**17**	24	–
10	**16**	24	0.14 ± 0.02
11	**15**	16	63.6 ± 2.3
12	**20**	14	10.2 ± 1.5
13	**19**	10	78.6 ± 2.5
14	**18**	24	–

According to the results shown in Table 2, phenols **9** and **10** cannot work as glycosyl acceptors in the transglucosylation reaction catalyzed by immobilized β-glucosidase with **3** as the glucosyl donor. These results are different from those reported by Yang et al. (Yang et al. 2012) and Tone et al. (Tong et al. 2004).

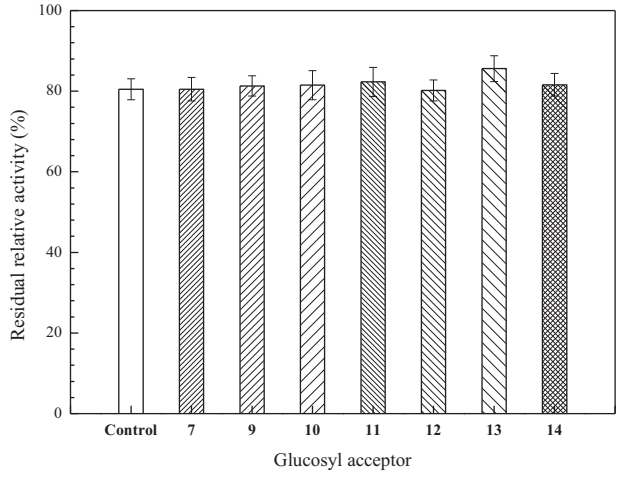


Figure 7. Activity of the immobilized β-glucosidase after incubating the enzyme in the *t*-butanol solution of **7**, **9**, **10**, **11**, **12**, **13** and **14** respectively for 24 hours at 50 °C.

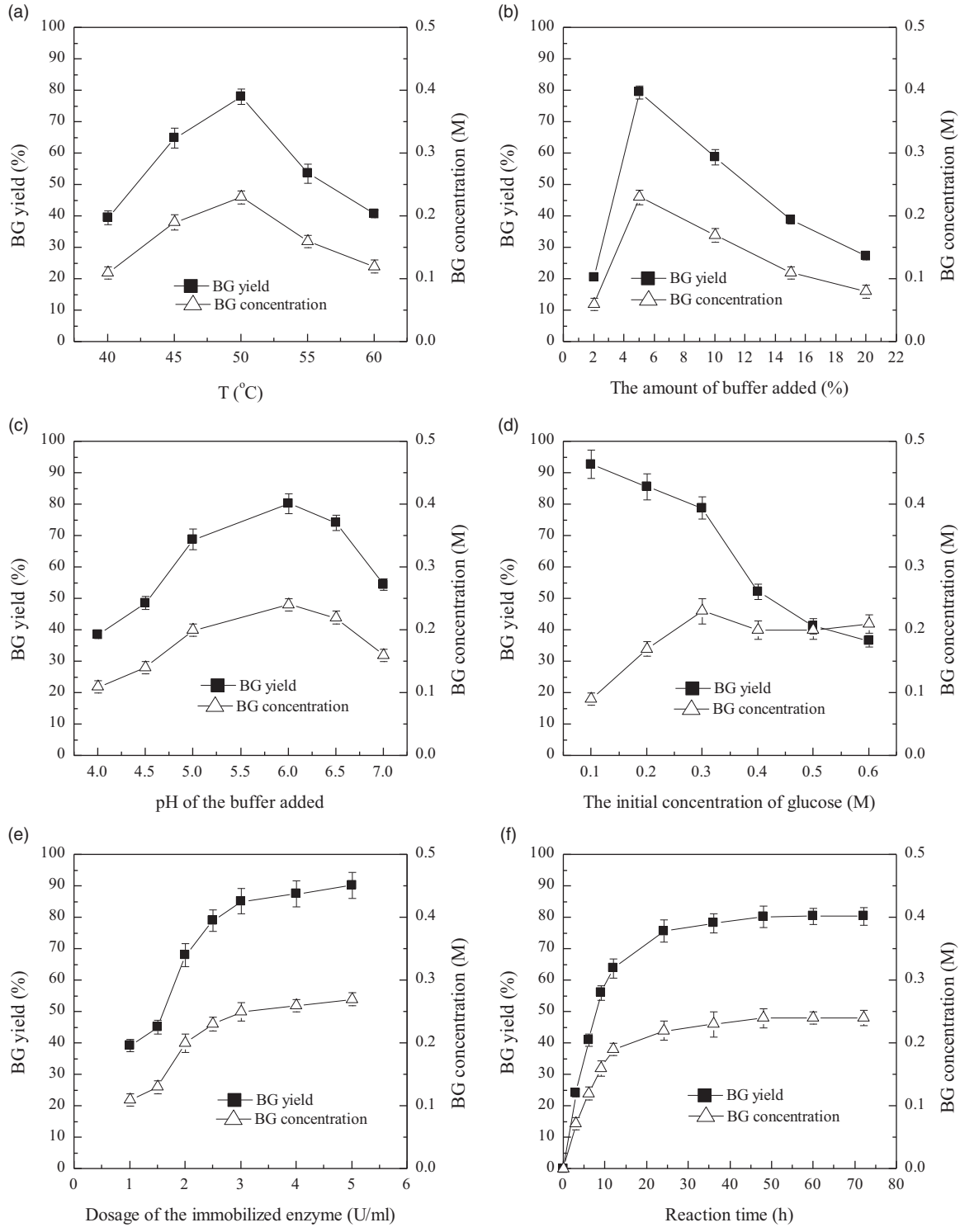


Figure 8. Effect of temperature (A), the amount of buffer added (B), pH of the buffer added (C), the initial concentration of glucose (D), the dosage of the immobilized enzyme (E) and the reaction time (F) on the BG synthesis through the reversed hydrolysis of the immobilized β-glucosidase.

They found that a yield of ~13.1%–28% can be obtained by the glucosylation of phenols catalyzed by *Prunus domestica* glucosidase or β-glucosidase extracted from a crude meal of apple seeds. Therefore, differences in the enzyme source, reaction medium, type of glucosyl donor, and immobilization method can influence the yield of glucosides produced via enzymatic synthesis (Rye and Withers 2000).

BG synthesized by the reverse hydrolysis of immobilized β-glucosidase

Compared to chemical methods, enzymatic synthesis of alkyl glucosides has several advantages, such as the anomeric purity of the product, reaction simplicity, and its eco-friendly nature (Rather and Mishra 2013). Reversed hydrolysis by glycosidases, the condensation reaction of a monosaccharide and an alcohol, in which water is the leaving group (reaction 1), was first reported in 1913 (Van Rantwijk et al. 1999). Here, the synthesis of the glucosyl donor **3** by the reverse hydrolysis of EP113/S with immobilized β-glucosidase was investigated and the results are shown in Figure 8. The enzymatic synthesis of **3** relies on a thermodynamic approach in which the equilibrium of the reaction, normally favoring hydrolysis, is shifted towards synthesis. Water acts as a competing nucleophile that causes parasitic hydrolysis of BG. Under the tested experimental conditions, the yield of **3** initially increased with an increase in the temperature, after which it decreased; the maximum yield of 78.04%±2.2% was obtained at 50 °C (Figure 8(A)). The reduction in yield at higher temperatures may be due to enzyme inactivation (Figure 1) and accelerated parasitic hydrolysis.

The synthesis of alkyl glycoside is generally hampered by the low solubility of carbohydrates in organic media (Van Rantwijk et al. 1999). Water is the preferred solvent for carbohydrates and to maintain the active conformation of β-glucosidase, an adequate amount of water is necessary. However, water can cause undesirable hydrolysis during the reverse hydrolysis of β-glucosidase; therefore, its concentration should be kept as low as possible (Halling 1994). Figure 8(B) demonstrates that the yield of **3** was the maximum when the content of water in the reaction mixture was 5% (v/v). The yield of **3** decreased when the water content was reduced to 2%. This is because the too small a water amount cannot ensure the active conformation of the enzyme (Vic et al. 1995). In addition, increasing the water content from 2% to 5% can increase the solubility of the carbohydrate, which is favorable for synthesis (Van Rantwijk et al. 1999). The active sites of β-glucosidase contain two carboxylic acid residues (Glu/Asp). One residue acts as an acid catalyst and the other acts as a base catalyst (Rather and Mishra 2013). In our study, we found that the optimal pH for the synthesis of **3** by immobilized β-glucosidase was 6.0 (Figure 8(C)). This observation differs from that of Vic et al. (Vic et al. 1995). They found that the pH of the water added to the reaction medium had no effect on the yield of the glucosylation reaction catalyzed by free almond β-glucosidase. This difference is may be due to differences in the composition of the media.

Because the reverse hydrolysis of β-glucosides can be inhibited by high glucose concentrations (Reshmi and Sugunan 2013; Wang et al. 2017), the influence of the initial concentration of glucose (0.1 M–0.6 M) on the synthesis of **3** catalyzed by immobilized β-glucosidase was investigated, as shown in Figure 8(D). The reaction was carried out in the presence of 2.5 U of immobilized β-glucosidase per mL of the medium (2.5 U/mL) and 5% (v/v) water/*n*-butanol at pH 6.0 and 50 °C. The yield of **3** decreased with an increase in the initial glucose concentration, while the amount of **3** produced leveled off at 0.3 M glucose.

Figure 8(E) illustrates the influence of the dosage of immobilized β-glucosidase on the yield and the amount of **3**. The reaction was carried out in the presence of 0.3 M glucose and 5% (v/v) water/*n*-butanol at pH 6.0 and 50 °C. The yield and amount of **3** produced increased by more than two-fold when the dosage of immobilized β-glucosidase was increased from 1 U/mL to 2.5 U/mL. However, doubling the dosage of the enzyme once again, i.e. from 2.5 U/mL to 5 U/mL, resulted in only a slight increase in both the yield and amount of **3**. An investigation on the time course of **3** syntheses was carried out and the results are shown in Figure 8(F). The yield of **3** plateaued in 24 h, reaching 0.23 ± 0.015 M of **3**.

Conclusions

Using BG as the glucosyl donor for salidroside synthesis via the transglucosylation of β-glucosidase of bitter almonds immobilized on EP113/S beads, a product yield of 39.04%±1.25% could be achieved with a corresponding BG product concentration of (0.59 ± 0.02) M. It was found that the glucoside yields decreased significantly when the hydroxyl groups of the glucosyl acceptor were phenolic in nature. Thus, immobilized β-glucosidase selectively catalyzed the glycosylation of the primary alcoholic hydroxyl groups of tyrosol with BG as the glucosyl donor. The synthesis of BG via the reversed hydrolysis of immobilized β-glucosidase resulted in a yield as high as 78.04%±2.2%. Salidroside synthesis catalyzed by enzymatic transglucosylation with BG as the glucosyl donor is a new enzymatic method for obtaining salidroside. In the future, screening of activated glycosyl donors and studies on more efficient and reusable enzymes should be carried out to improve the efficiency of salidroside synthesis via enzymatic transglycosylation reactions.

Disclosure statement

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

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References

Akita H, Kawahara E, Kishida M, Kato K. 2006. Synthesis of naturally occurring β-D-glucopyranoside based on enzymatic β-glycosidation. J Mol Catal B Enzym. 40:8–15.

Akita H, Kurashima K, Nakamura T, Kato K. 1999. Chemoenzymatic syntheses of naturally occurring β-glucosides. Tetrahedron: Asymmetry. 10:2429–2439.

Andersson M, Adlercreutz P. 2001. A kinetic study of almond-β-glucosidase catalyzed synthesis of hexyl-glycosides in low aqueous media: influence of glycosyl donor and water activity. J Mol Catal B Enzym. 14:69–76.

Chen L, Hu Y-D, Li N, Zong M-H. 2012. Cross-linked enzyme aggregates of β-glucosidase from *Prunus domestica* seeds. Biotechnol Lett. 34:1673–1678.

Crout DHG, Vic G. 1998. Glycosidases and glycosyl transferases in glycoside and oligosaccharide synthesis. Curr Opin Chem Biol. 2:98–111.

Delépée R, Berteina-Raboin S, Lafosse M, Lamy C, Darnault S, Renimel I, About N, Andre P. 2007. Synthesis, purification, and activity of salidroside. J Liq Chromatogr Relat Technol. 30:2069–2080.

Deslongchamps P. 1993. Intramolecular strategies and stereoelectronic effects. Glycosides hydrolysis revisited. Pure Appl Chem. 65:1161–1178.

Dougherty DA. 1996. Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. Science. 271:163.

Drueckhammer DG, Hennen WJ, Pederson RL, Iii CFB, Gautheron CM, Krach T, Wong C-H. 1991. Enzyme catalysis in synthetic carbohydrate chemistry. Synthesis. 1991:499–525.

Esen A. 1993. Chapter 1, β-Glucosidases. In: Esen A, editor. ß-Glucosidases. Washington, DC: American Chemical Society; p.1–14.

Fernandez-Lafuente R. 2009. Stabilization of multimeric enzymes: strategies to prevent subunit dissociation. Enzyme Microb Technol. 45:405–418.

González-Pombo P, Fariña L, Carrau F, Batista-Viera F, Brena BM. 2011. A novel extracellular β-glucosidase from Issatchenkia terricola: isolation, immobilization and application for aroma enhancement of white Muscat wine. Process Biochem. 46:385–389.

Guo Y, Zhao Y, Zheng C, Meng Y, Yang Y. 2010. Synthesis, biological activity of salidroside and its analogs. Chem Pharm Bull. 58:1627–1629.

Gupta MN. 1993. Chapter 307, Biocatalyst design for stability and specificity. In: Himmel ME, Georgiou G, editors. ACS Symposium Series. Washington: ACS Publications.

Halling PJ. 1994. Thermodynamic predictions for biocatalysis in nonconventional media: theory, tests, and recommendations for experimental design and analysis. Enzyme Microb. Technol. 16:178–206.

Ismail A, Soltani S, Ghoul M. 1998. Optimization of the enzymatic synthesis of butyl glucoside using response surface methodology. Biotechnol Progr. 14:874–878.

Jiang J, Yin H, Wang S, Zhuang Y, Liu S, Liu T, Ma Y. 2018. Metabolic Engineering of *Saccharomyces cerevisiae* for high-level production of salidroside from glucose. J Agric Food Chem. 66:4431–4438.

Jonathan W, 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.

Karagulyan HK, Gasparyan VK, Decker SR. 2008. Immobilization of fungal beta-glucosidase on silica gel and kaolin carriers. Appl Biochem Biotechnol. 146:39–47.

Kharrat N, Ali YB, Marzouk S, Gargouri Y-T, Karra-Châabouni M. 2011. Immobilization of *Rhizopus oryzae* lipase on silica aerogels by adsorption: comparison with the free enzyme. Process Biochem. 46:1083–1089.

Klibanov AM. 1997. Why are enzymes less active in organic solvents than in water? Trends Biotechnol. 15:97–101.

Koshland DE. 1953. Stereochemistry and the mechanism of enzymatic reactions. Biol Rev. 28:416–436.

Laane C, Boeren S, Vos K, Veeger C. 1987. Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng. 30:81–87.

Li Y, Pham V, Bui M, Song L, Wu C, Walia A, Uchio E, Smith-Liu F, Zi X. 2017. *Rhodiola rosea L.*: an Herb with anti-stress, anti-aging, and immunostimulating properties for cancer chemoprevention. Curr Pharmacol Rep. 3:384–395.

Lu W-Y, Lin G-Q, Yu H-L, Tong A-M, Xu J-H. 2007. Facile synthesis of alkyl β-d-glucopyranosides from D-glucose and the corresponding alcohols using fruit seed meals. J Mol Catal B Enzym. 44:72–77.

Lundemo P. 2015. Transglycosylation by Glycoside Hydrolases-Production and modification of alkyl glycosides [Doctoral thesis]. Sweden: Lund University.

Makropoulou M, Christakopoulos P, Tsitsimpikou C, Kekos D, Kolisis FN, Macris BJ. 1998. Factors affecting the specificity of β-glucosidase from *Fusarium oxysporum* in enzymatic synthesis of alkyl-β-D-glucosides. Int J Biol Macromol. 22:97–101.

Mateo C, Abian O, Fernandez-Lorente G, Pedroche J, Fernandez-Lafuente R, Guisan JM, Tam A, Daminati M. 2002. Epoxy sepabeads: a novel epoxy support for stabilization of industrial enzymes via very intense multipoint covalent attachment. Biotechnol Prog. 18:629–634.

Mateo C, Grazu V, Palomo JM, Lopez-Gallego F, Fernandez-Lafuente R, Guisan JM. 2007a. Immobilization of enzymes on heterofunctional epoxy supports. Nat Protoc. 2:1022–1033.

Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. 2007b. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb Technol. 40:1451–1463.

Matsumura S, Imai K, Yoshikawa S, Kawada K, Uchibor T. 1990. Surface activities, biodegradability and antimicrobial properties of n-alkyl glucosides, mannosides and galactosides. J Am Oil Chem Soc. 67:996–1001.

Najafpour GD. 2007. Biochemical Engineering and Biotechnology. Amsterdam: Elsevier. Chapter 5, Growth Kinetics; p.81–141.

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.

Potocká E, Mastihubová M, Mastihuba V. 2015. Enzymatic synthesis of tyrosol glycosides. J Mol Catal B Enzym. 113: 23–28.

Reshmi R, Sugunan S. 2013. Improved biochemical characteristics of crosslinked β-glucosidase on nanoporous silica foams. J Mol Catal B Enzym. 85:111–118.

Rather MY, Mishra S. 2013. β-Glycosidases: an alternative enzyme-based method for synthesis of alkyl-glycosides. Sustain Chem Process. 1:1.

Rodrigues RC, Ortiz C, Berenguer-Murcia Á, Torres R, Fernández-Lafuente R. 2013. Modifying enzyme activity and selectivity by immobilization. Chem Soc Rev. 42: 6290–6307.

Roig MG, Estevez FB, Velasco FG, Ghais NI, Cachaza Silverio JM. 1986. Biotechnology and applied biology section: methods for immobilizing enzymes. Biochem Educ. 14: 180–185.

Rye CS, Withers SG. 2000. Glycosidase mechanisms. Curr Opin Chem Biol. 4:573–580.

Shi T, Chen H, Jing L, Liu X, Sun X, Jiang R. 2011. Development of a kilogram-scale synthesis of salidroside and its analogs. Synth Commun. 41:2594–2600.

Shi T-y, Feng S-f, Xing J-h, Wu Y-m, Li X-q, Zhang N, Tian Z, Liu S-b, Zhao M-g. 2012. Neuroprotective effects of salidroside and its analog tyrosol galactoside against focal cerebral ischemia *in vivo* and H_2O_2-induced neurotoxicity *in vitro*. Neurotox Res. 21:358–367.

Singh RK, Zhang Y-W, Nguyen N-P-T, Jeya M, Lee J-K. 2011. Covalent immobilization of β-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles. Appl Microbiol Biotechnol. 89:337–344.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano M, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem. 150:76–85.

Stambaugh R, Post D. 1966. A substrate and product inhibition of rabbit muscle lactic dehydrogenase heart (H4) and muscle (M4) isozymes. J Biol Chem. 241:1462–1467.

Tong AM, Lu WY, Xu JH, Lin GQ. 2004. Use of apple seed meal as a new source of β-glucosidase for enzymatic glucosylation of 4-substituted benzyl alcohols and tyrosol in a monophasic aqueous-dioxane medium. Bioorg Med Chem Lett. 14:2095–2097.

Tong A-M, Xu J-H, Lu W-Y, Lin G-Q. 2005. Construction and optimization of a monophasic organic–water system for enzymatic synthesis of p-nitrobenzyl β-d-glucopyranosides by reverse hydrolysis. J Mol Catal B Enzym. 32:83–88.

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.

Urata K, Yano S, Takaishi N. 1995. Preparations and reactions of alkyl ethers of some aldohexoses. J Am Oil Chem Soc. 72:73–81.

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 JM, Thomas D. 1995. Enzymatic glucosylation of hydrophobic alcohols in an organic medium by the reverse hydrolysis reaction using almond-b-D-glucosidase. Biotechnol Bioeng. 46:109–116.

von Rybinski W, Hill K. 1998. Alkyl polyglycosides-properties and applications of a new class of surfactants. Angew Chem Int Ed Engl. 37:1328–1345.

Wang F, Ma Y, Liu Y-H, Zhang X, Zhang F, Linhardt RJ. 2017. Improved octyl glucoside synthesis using immobilized β-glucosidase on PA-M with reduced glucose surplus inhibition. Biocatal Biotransform. 35:349–362.

Wang S, Meng X, Zhou H, Liu Y, Secundo F, Liu Y. 2016. Enzyme stability and activity in non-aqueous reaction systems: a mini review. Catalysts. 6:32.

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.

Woudenberg-van Oosterom M, van Belle HJA, van Rantwijk F, Sheldon RA. 1998. Immobilized β-galactosidases and their use in galactoside synthesis. J Mol Catal A Chem. 134:267–274.

Wu S, Zu Y, Wu M. 2003. High yield production of salidroside in the suspension culture of *Rhodiola sachalinensis*. J Biotechnol. 106:33–43.

Yang R-L, Li N, Zong M-H. 2012. Using ionic liquid cosolvents to improve enzymatic synthesis of arylalkyl β-d-glucopyranosides. J Mol Catal B Enzym. 74:24–28.

Yang XP, Wang FF, Yan J, Ma K, Mao DB. 2017. A new family-3 glycoside hydrolase from *Penicillium oxalicum* BL 3005 catalyzing tyrosol glucosylation to form salidroside. Biotechnol Appl Biochem. 64:525–531.

Yanhong BI, Zhaoyu W, Yanyong MAO, Zheng S, Zhang H, Hao SHI. 2012. Ionic liquid effects on the activity of β-glycosidase for the synthesis of salidroside in co-solvent systems. Chin J Catal. 33:1161–1165.

Yu H-L, Xu J-H, Lu W-Y, Lin G-Q. 2007. Identification, purification and characterization of β-glucosidase from apple seed as a novel catalyst for synthesis of O-glucosides. Enzyme Microb Technol. 40:354–361.

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.

Zhang J-X, Ma L-Q, Yu H-S, Zhang H, Wang H-T, Qin Y-F, Shi G-L, Wang Y-N. 2011. A tyrosine decarboxylase catalyzes the initial reaction of the salidroside biosynthesis pathway in *Rhodiola sachalinensis*. Plant Cell Rep. 30:1443–1453.

Zhang C, Yu H, Lu M, Li J, Jin F. 2005. Enzymic synthesis of salidroside: purification and characterization of salidrosidase from *Aspergillus niger*. Process Biochem. 40: 3143–3147.