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IMMOBILIZATION OF PUERARIN GLYCOSIDASE FROM *Microbacterium oxydans* CGMCC 1788 INCREASES PUERARIN TRANSFORMATION EFFICIENCY

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Abstract - For immobilization of puerarin glycosidase from *Microbacterium oxydans* CGMCC 1788 on DEAE-52 cellulose, the optimal amount of enzyme protein was 12 mg protein: 1 g DEAE-52 cellulose; the optimal pH was 6.5; and the optimal immobilization time was 6 hr. The specific activity of immobilized enzyme was 36.67 $mU.g^{-1}$ carrier with an immobilization yield of 98.87% and an enzyme recovery yield of 92.43%. The molar transformation rates of puerarin by immobilized enzyme and by the relative bacterial cell amount equal to the same amount of enzyme were 53.3% and 2.2%, respectively, after 1 hr of transformation. The former molar transformation rate, which was similar to that for free enzyme, was more than 24-fold greater than the latter. The immobilized puerarin glycosidase showed improved enzymatic properties and stability. The immobilized puerarin glycosidase retained 88% of its initial activity after being reused 10 times.
Keywords: Puerarin; Immobilized enzyme; Puerarin-7-*O*-Glucoside, *Microbacterium oxydans*; DEAE-52 Cellulose.

INTRODUCTION

Puerarin, an isoflavone glycoside isolated from the roots of *Puerarin thomsonii* and *Pueraria lobata*, has been reported to treat cardio-cerebrovascular disease, diabetes, ocular fundus disease, sudden deafness and acute alcoholic poisoning (Benlhabib *et al*., 2004). However, the pharmacological development of puerarin is limited by its low water solubility and bioavailability (Ren *et al*., 2006). Thus, significant effort has been made to chemically or biologically modify the structure of puerarin to obtain novel puerarin derivatives with higher water solubility and biological activity (Li *et al*., 2004; Huang *et al*., 2008; Jiang *et al*., 2008; Yu *et al*., 2010; Ko *et al*., 2012). Previous studies have shown that transformation of flavonoid substances in the form of aglycon to glycosides improves not only their physical and chemical properties, such as water solubility, taste and sweetness but also their pharmacological activities, including circulation, metabolism, and concentration in body fluids (Lee *et al*., 1999; Kren *et al*., 2001; Daines *et al*., 2004; Blanchard *et al*., 2006; Hyung *et al*., 2006; Salas *et al*., 2007).

Jiang *et al*. (2008) and Yu *et al*. (2010) from our laboratory have previously reported that puerarin is transformed to puerarin-7-*O*-glucoside and puerarin-7-*O*-fructoside, respectively, under different condi-

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tions by *Microbacterium oxydans* (*M. oxydans*) CGMCC 1788, and the transformation effectively increases the water solubility of puerarin. However, the transformation of puerarin by resting cells is limited by the low water solubility of puerarin and the barrier of the cell membrane, leading to low transformation efficiency, long reaction time, and many by-products. Although crude enzyme cell extract may be used to increase transformation efficiency and shorten the reaction period by removing the barrier of the cell membrane and promoting direct interaction between the enzyme and the substrate, it is difficult to isolate crude enzymes from products and reuse them for transformation.

Immobilized enzyme has a lot of advantages over free enzyme including improved storage stability and easy retrieval for re-use (Brady *et al*., 2009; Sheldon *et al*., 2013). Also, immobilization enhances enzyme specificity and activity (Pollard *et al*., 2007; Woodley, 2008; Garcia-Galan *et al*., 2011), reduces the possibility of contamination by microbes (Singh, 2008), decreases the cost of continuous production, and improves purity of the final products (D'Souza, 1999). Indeed, studies of immobilized enzymes have advanced tremendously since Tosa *et al*. (1967) first utilized immobilized aminoacylase to achieve continuous industrial production of L-amino acids in the 1960s (Tosa *et al*., 1967; Xie *et al*., 2009; Abdelmajeed *et al*., 2012; Vlakh *et al*., 2013; Contesini *et al*., 2013). In this study, we utilized DEAE-52 cellulose as the carrier to adsorb and immobilize puerarin glycosidase extracted from *M. oxydans* CGMCC 1788 to transform puerarin. Our study provides a new approach for industrial production of puerarin glycosides.

MATERIALS AND METHODS

Materials

Puerarin (98% purity) was purchased from Jiangsu LianChuang Pharmaceutical Co., Ltd (Nanjing, China); Bradford solution, BSA solution, methanol and ethanol (HPLC grade) were purchased from Sigma (St. Louis, USA); DEAE-52 cellulose, AB-8 macro-porous resin was purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China).

Bacteria Culture

M. oxydans CGMCC 1788 was streaked on a LB plate and grown at 30 °C overnight. One single colony was inoculated in 20 mL LB medium in a 100 mL conical flask and grown at 30 °C, 220 rpm for 24 hr. 1% of the 20 mL culture was inoculated into 1 liter of LB medium in a 3-liter conical flask and grown at 30 °C, 220 rpm for 12 hr to an OD600 of 2.0-3.0.

Preparation of Crude Enzyme Extract and Measurement of Protein Concentration

1 liter of bacteria culture was centrifuged at 4 °C, 8000 × g for 10 min. The bacterial pellet was re-suspended in 200 mL of pre-chilled 1/15 M Na_2HPO_4/KH_2PO_4 buffer (PBS, pH 6.5) and disrupted for 2 × 1 min at 1 min intervals with a Bead-beater (BioSpecs Product, USA) on ice by using 0.1 mm glass beads (BioSpecs Product, USA). The cell lysate was centrifuged at 4 °C, 12000 × g for 10 min. After discarding the cellular debris, 180 ml of crude enzyme extract was obtained. The protein concentration of the crude enzyme extract was determined by the Bradford method (Bradford, 1976).

Immobilization of Enzyme

DEAE-52 cellulose was pretreated with 0.5 M HCl and 0.5 M NaOH, respectively, and then washed with deionized water until neutral pH. Crude enzyme extract in PBS buffer (pH 6.5) was mixed with DEAE-52 cellulose at a 12 mg: 1 g (enzyme: DEAE-52 cellulose) ratio, and the mixture was constantly stirred for full adsorption at 4 °C for 6 hr. After removing the supernatant, the DEAE-52 cellulose-immobilized enzyme was washed twice with PBS buffer and dried by vacuum suction. To study the effect of enzyme amount on the immobilization rate, crude enzyme extract was added to DEAE-52 cellulose at 4-20 mg: 1 g (enzyme: DEAE-52 cellulose) ratios; to study the effect of pH on the immobilization rate, PBS buffer ranging from pH 5 to 9 was tested; to study the effect of adsorption time on the immobilization rate, DEAE-52 cellulose was incubated with enzyme extract for 2-24 hr.

Measurement of Enzyme Activity

Enzyme activity was determined by measuring the amount of puerarin-7-*O*-glucoside produced by puerarin glycosidase. One unit of enzyme activity (1 U) was defined as the enzyme amount that catalyzes the formation of 1 μmol puerarin-7-*O*-glucoside in 1 min. To measure the free enzyme activity, 6 mg of free enzyme was added to 10 mL of 1/15 M PBS buffer (pH 6.5) containing sucrose (20 mg/mL) and puerarin (2 mg/ml) and incubated at 30 °C,

220 rpm for 1 hr. The mixture was heated to 100 °C for 10 min to stop the reaction, then centrifuged at 12,000 × g for 10 min and the supernatant was collected for HPLC analysis. To measure the immobilized enzyme activity, 0.5 g of dried DEAE-52 cellulose-immobilized enzyme (equal to 6 mg free enzyme) was added to 10mL of 1/15 MPBS buffer (pH 6.5) containing sucrose (20 mg/mL) puerarin (2 mg/mL) and incubated at 30 °C, 220 rpm for 1 hr. The mixture was heated to 100 °C for 10 min to stop the reaction, then centrifuged at 12,000 × g for 10 min and the supernatant was collected for HPLC analysis. To measure the effect of different substrate concentrations on transformation rates of the immobilized enzyme, the puerarin concentrations were varied from 0.2 to 4 mg/mL; to measure the effect of temperature on the transformation rate; the reaction temperature was controlled within 10-60 °C. To measure the effect of pH on the transformation rate, the pH of the reaction ranged from 4-9. To measure the kinetic constant *K*m, the enzymatic reaction time was set to 5, 10, 15, 20 min, respectively, and the substrate concentration was set to 0.5, 1, 1.5, 2.0 mg/mL for free enzyme and 0.5, 1, 1.5, 2.0. 2.5 mg/mL for immobilized enzyme, respectively.

Small-Scale Transformation of Puerarin by Immobilized Enzyme

12 g of puerarin were first dissolved in 3 L of 1/15 M PBS buffer (pH 6.5) with constant stirring, then mixed with 50 g of dried immobilized enzyme in a GBCS-5B-1 5 L fermenter (Zhenjiang East Biotech Equipment and Technology Co., Ltd, China), and incubated at 30 °C, 500 rpm for 12 hr. The reaction mixture was centrifuged at 8,000 × g for 10 min and the supernatant was heated to 100 °C for 10 min and centrifuged again at 12,000 × g for 10 min. The final supernatant was applied to an AB-8 macroporous resin adsorption chromatography column for further separation and purification (Yu *et al*., 2010). Each collected fraction was subjected to HPLC analysis and the fractions of > 95% purity of the transformation product were pooled, concentrated to 40-50 ml in a rotary evaporator, and freeze-dried to obtain the powder.

Identification and Analysis of Transformation Product by HPLC

The transformation product, puerarin-7-*O*-glucoside, was qualitatively and quantitatively analyzed using an Agilent 1100 HPLC (Agilent, USA) as described by Ye *et al*. (2007). Characterization of the structure of the product was carried out as described by Yu *et al*. (2010).

RESULTS

Optimizing Conditions of Enzyme Immobilization

Effect of Enzyme Amount on Immobilization

As shown in Table 1, the activity of immobilized enzyme increased with increasing amount of enzyme. However, when the amount of enzyme was ≥ 16 mg.g^{-1} DEAE-52 cellulose, the binding sites on the surface of the DEAE-52 support tended to saturation and its capacity to bind protein started to decrease, so the recovery yield of enzyme activity decreased significantly to 76.19%. Considering the immobilization yield together with the recovery yield of enzyme activity, the optimal amount of enzyme was 12 mg.g^{-1} DEAE-52 cellulose. Under this condition, the specific activity of the immobilized enzyme was 36.97 mU.g^{-1}, the recovery yield of the enzyme activity reached 92.43% and the immobilization yield was 98.87%.

Table 1: Effect of enzyme amount on immobilization.

Added enzyme (mg protein.g-1 carrier)	Added enzyme (*A*) (mU. g^{-1} carrier)	Unbound enzyme (*U*) (mU.g^{-1} carrier)	Immobilized enzyme (*I*) (mU.g^{-1} carrier)	Recovery yield of enzyme (%) (R)=I/A×100	Immobilization yield (%) (*Y*)=*I*/(*A*-*U*)×100
4	9.00±0.38	0.02±0.003	9.00±0.21	100.00	100.22
8	21.33±1.39	0.83±0.053	20.00±2.28	93.76	97.56
12	40.00±3.16	2.68±0.128	36.97±3.38	92.43	98.87
16	70.00±4.26	15.75±1.12	53.33±4.22	76.19	98.30
20	106.67±3.72	41.39±4.19	60.00±3.24	56.25	91.91

Effect of Adsorption pH on Enzyme Immobilization

As shown in Figure 1, the optimal pH is 6.5 for enzyme immobilization, and the adsorption of puerarin glycosidase to DEAE-52 cellulose decreased when the pH was higher or lower than 6.5. This reduction of adsorption to DEAE-52 cellulose at pH≥7.0 may be related to the chemical structure of puerarin glycosidase. Different pH results in a difference of the charge distribution on the surface of the enzyme and therefore affects its binding to the support.

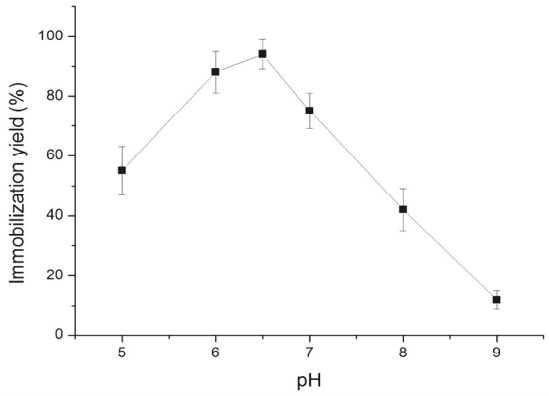

Figure 1: Effect of adsorption pH on enzyme immobilization.

Effect of Adsorption Time on Enzyme Immobilization

As shown in Figure 2, the immobilization yield of the enzyme gradually increased with prolonged adsorption time. But the yield did not increase further after adsorption for more than 6 hr, suggesting that the adsorption of puerarin glycosidase to DEAE-52 cellulose is complete after 6 hr. Therefore, the optimal adsorption time for enzyme immobilization is 6 hr.

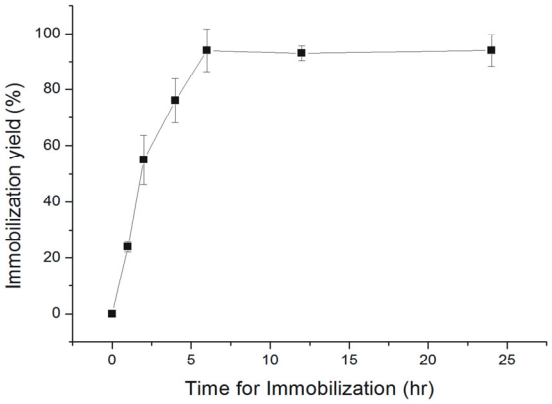

Figure 2: Effect of adsorption time on enzyme immobilization.

Transformation Efficiency of Immobilized Enzyme

Ten mL of bacteria culture with an OD600 of 2.0 was disrupted to prepare free or immobilized enzyme and the same amount of bacterial culture was used as the control for the transformation reaction (Figure 3). After reaction for 1 hr, the molar transformation rates of immobilized enzyme, free enzyme and bacterial cells were 53.3%, 66.2% and 2.2%, respectively. Therefore, the transformation efficiencies of immobilized enzyme and free enzyme were 24.2 and 30.1 fold greater than that of control bacterial cells, respectively. Considering the recovery yield of immobilized enzyme to be 92.43%, the actual molar transformation rate of immobilized enzyme was similar to that of free enzyme. After 24-hr of the transformation reaction, the transformation activities of immobilized enzyme and free enzyme were still more than 2-fold greater than that of control bacterial cells.

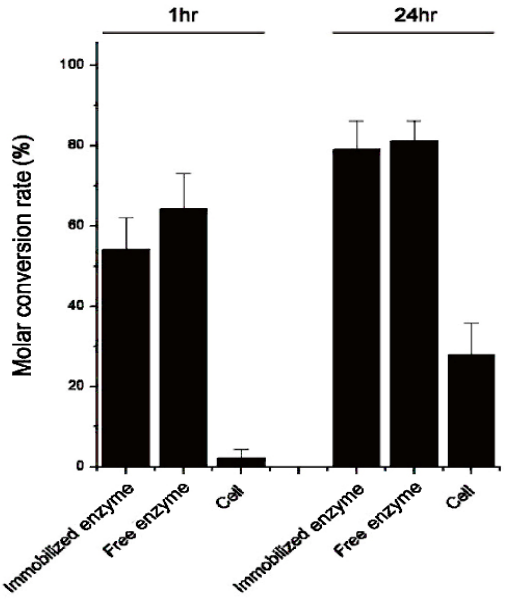

Figure 3: Comparison of transformation activity for immobilized enzyme, free enzyme and control bacterial cells.

Enzymatic Properties of Immobilized Enzyme

Optimal Substrate Concentration for Immobilized Enzyme

As shown in Figure 4, the activity of immobilized enzyme increased with the increase of substrate concentration from 0.2 to 2 mg/mL. When the substrate concentration exceeded 2 mg/mL, the activity of

immobilized enzyme no longer increased. However, the activity of free enzyme still increased as the substrate concentration increased to 4 mg/mL.

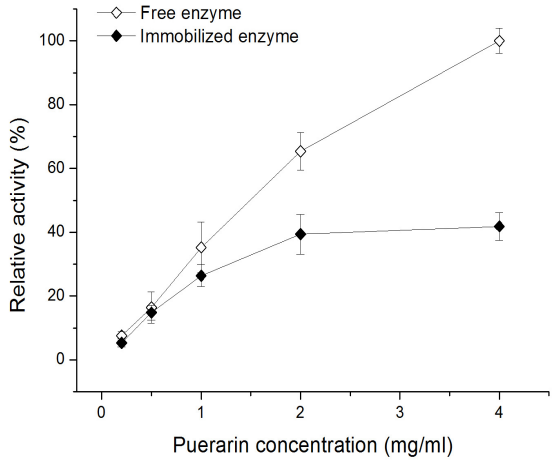

Figure 4: The effect of puerarin concentration on the biotransformation activity of free enzyme and immobilized enzyme.

Optimal Reaction Temperature for Immobilized Enzyme

As shown in Figure 5, the optimal reaction temperatures were 40 °C and 35 °C for free enzyme and immobilized enzyme, respectively. The immobilized enzyme retained a higher activity at 30-40 °C, while the activity of free enzyme was lower than that of immobilized enzyme at 30-35 °C, implying that substrate molecules diffuse more easily on the molecular surface of enzyme immobilized on the support compared to free enzyme. When the temperature was over 40 °C, the transformation activity of both immobilized enzyme and free enzyme decreased significantly, and they were completely inactivated at 50 °C, indicating that, with the increase of temperature, the conformations of both immobilized and free enzyme molecules opened up so they lost their catalytic activity.

Optimal pH for Immobilized Enzyme

As shown in Figure 6, the optimal pH of the transformation reaction for both immobilized and free enzyme was 6.5. However, the immobilized enzyme retained its higher activity at pH≥6.5 while free enzyme retained its higher activity at pH≤6.5. These small changes of enzymic activity with pH may be attributed to the effect of the negatively-charged support for enzyme immobilization which will result in a basic shift in the pH optimum (Goldstein *et al*., 1964; Shi *et al*., 2010).

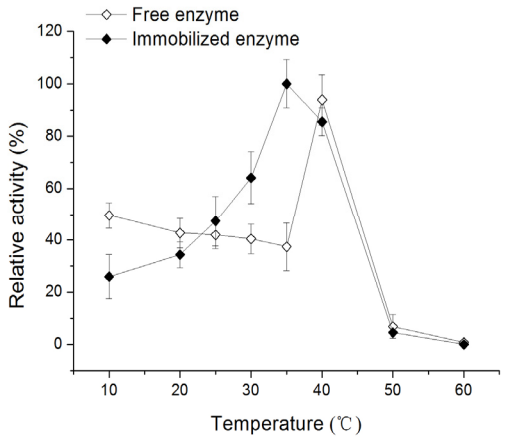

Figure 5: Effect of reaction temperature on the transformation activity of free enzyme and immobilized enzyme.

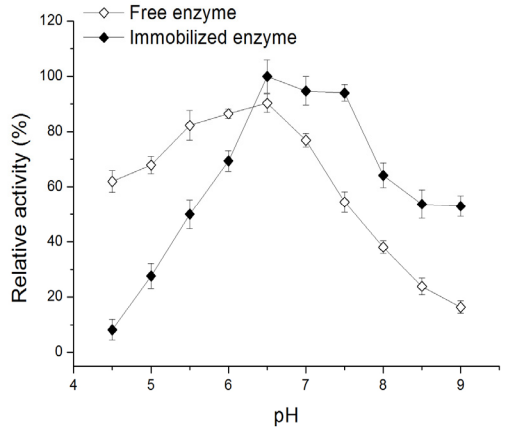

Figure 6: Effect of pH on the transformation activities of free enzyme and immobilized enzyme.

Kinetic Constant Km

Kinetic constants *K*m for the free and immobilized glucoamylase were determined using puerarin as the substrate and were calculated from Lineweaver–Burk plots as shown in Figure 7. *K*m was found to be 0.62 mM for the free enzyme and 3.94 mM for the immobilized enzyme. The value of *K*m for immobilized enzyme was approximately 6.4 times higher than that of the free enzyme. This increase may reflect the reduction of the affinity of the substrate

for the active site of the enzyme which resulted from conformational and steric modifications of the enzyme introduced by binding to DEAE-52 cellulose and the substrate transfer resistance inherent in the morphology of the support (Shi *et al*., 2010).

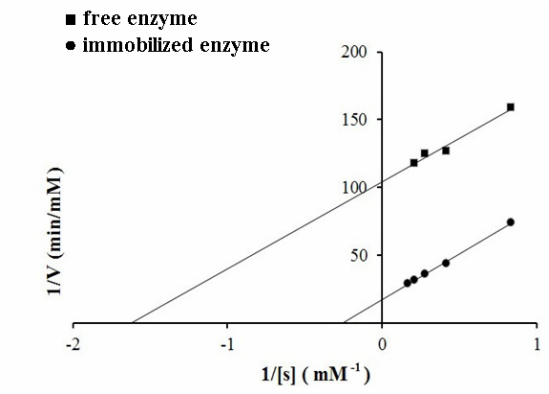

Figure 7: Lineweaver-Burk plots for the free enzyme and immobilized enzyme.

Thermal Stability of Immobilized Enzyme

The initial activities of free enzyme and immobilized enzyme prior to temperature treatment were set as the 100% control. Free enzyme and immobilized enzyme were kept at different temperatures for 30 min, then cooled in an ice water bath before the transformation reaction. The activities of enzymes were measured and compared to controls, as shown in Figure 8. Immobilized enzyme showed improved thermal stability since it retained 68% of its initial activity compared to the free enzyme, which only retained 9.5% of its initial activity after being incubated at 50 °C for 30 min. This thermal stability of immobilized enzyme may be due to improved stability of the three-dimensional structure of the immobilized enzyme compared to free enzyme (Karim *et al*., 2002).

Effect of pH on the Stability of Immobilized Enzyme

Free enzyme and immobilized enzyme were kept in different pH solutions for 30 min before the transformation reaction and their activities were then measured and compared to their initial activities. As shown in Figure 9, immobilized enzyme was more stable at pH≥6.5, while free enzyme was more stable at pH 5.5-6.0. Apparently, free puerarin glycosidase is not stable at higher pH, However, immobilization to DEAE-52 cellulose may stabilize the three-dimensional structure of the enzyme to protect the enzyme from denaturation at higher pHs.

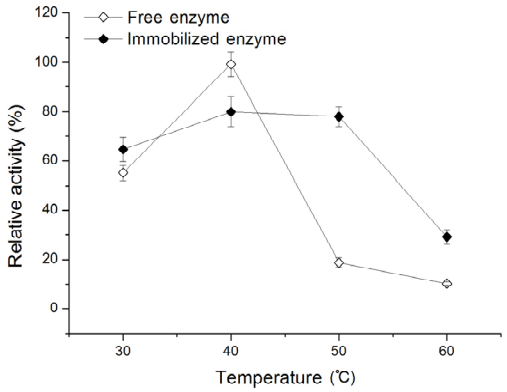

Figure 8: Comparison of thermal stability between free enzyme and immobilized enzyme

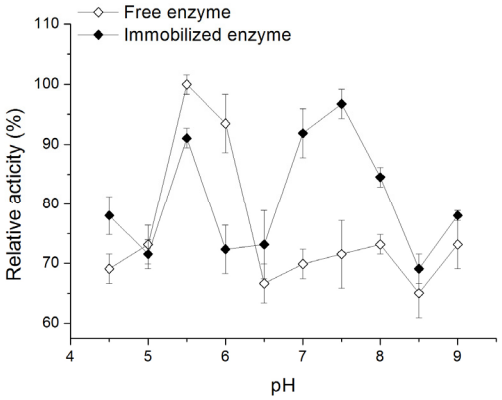

Figure 9: Effect of pH on the stability of free enzyme and immobilized enzyme.

Handling Stability of Immobilized Enzyme

Immobilized enzyme was continuously reused 10 times for the transformation assay and enzyme activity was measured after each use. The enzyme activity for the first reaction was set as the control of 100%. As shown in Figure 10, the immobilized enzyme retained 88% of its initial activity after being reused 10 times. This suggests that immobilized enzyme is stable for being reused multiple times.

Storage Stability of Immobilized Enzyme

Free enzyme and immobilized enzyme were stored at 4 °C and their enzyme activities were measured every 5 days for 45 days as shown in Figure 11. After being stored at 4 °C for 45 days, free enzyme

and immobilized enzyme retained 97.5% and 104.8%, respectively, of their initial transformation activities measured on day 0. The result suggests that immobilization improves the storage stability of the enzyme.

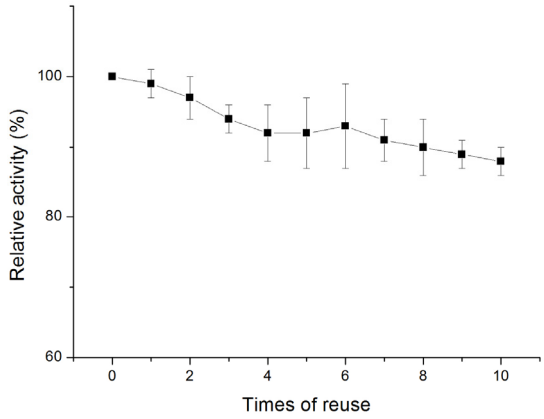

Figure 10: Stability of reused immobilized enzyme.

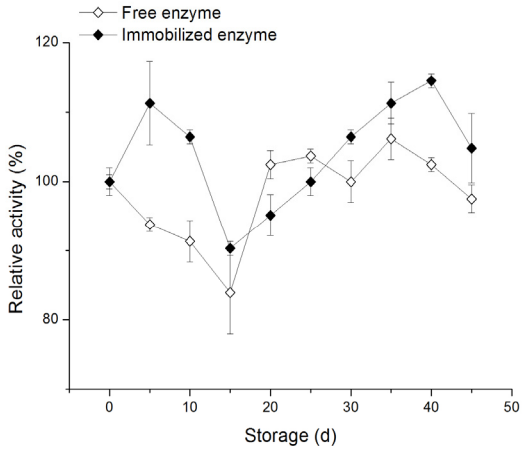

Figure 11: Storage stability of immobilized enzyme.

Small-Scale Transformation of Puerarin by Immobilized Enzyme

A 3L volume scale transformation under the above optimal conditions was conducted. When 12 g of puerarin was transformed by 50g of dry immobilized enzyme in 3 liter of 1/15 M PBS buffer (pH 6.5) for 12 hr, the molar transformation rate was 71.8%. The transformation product was separated, purified, condensed and identified as puerarin-7-*O*-glucoside, yielding 7.8 g of 96.8% purity (Figure 12). Therefore, the recovery yield of puerarin glycoside was 63.1% compared to the theoretical value of transformation product of 12.3 g.

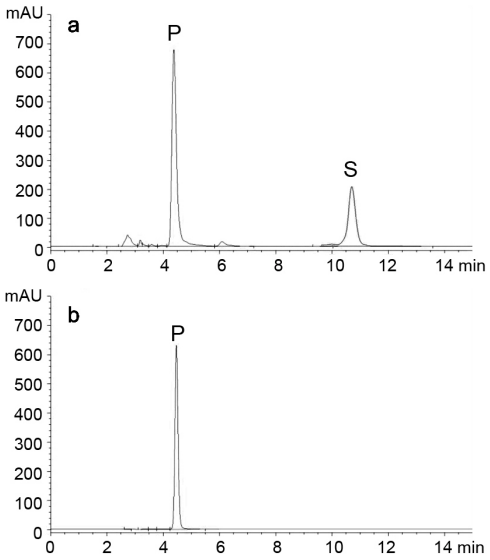

Figure 12: HPLC chromatograms of transformation of puerarin by immobilized enzyme in a fermenter. (a) Transformation solution. (b) The purified products from the transformation solution. P, the product. S, the substrate.

DISCUSSION

M. oxydans CGMCC 1788 is capable of transforming puerarin to glycosylated puerarin. However, the transformation by resting cells is limited by low transformation efficiency and long transformation time because extracellular puerarin diffuses slowly into the intracellular space through the barrier of the cell membrane. When the intracellular puerarin reaches a certain concentration, it is transformed by *M. oxydans* CGMCC 1788 glycosidase to glycosylated puerarin that is then secreted through the cell membrane to the extracellular solution (Jiang *et al*., 2008; Yu *et al*., 2010). In order to increase the transformation efficiency and shorten the transformation time, free glycosidase extracted from disrupted cells of *M. oxydans* CGMCC 1788 has been used to transform puerarin (Yu *et al*., 2010). Although the transformation efficiency of free enzyme is increased by its direct reaction with puerarin in solution without the barrier of the cell membrane, it is difficult to isolate free enzyme from products and reuse it for transformation, increasing the production cost (Yu *et al*., 2010). In this study, we reported that the transformation efficiency is significantly increased by immobilized puerarin glycosidase. Indeed, immobilized

enzyme and free enzyme showed similar transformation efficiencies that are at least 24-fold greater than that of resting cells. Notably, immobilized enzyme can be recycled and reused. As shown in this study, DEAE-52 cellulose-immobilized enzyme retains 88% of the initial transformation activity after being reused 10 times. This suggests that immobilized enzyme is stable during handling and it rarely detaches from the DEAE-52 cellulose during transformation reactions. Therefore, this immobilization method is particularly suitable for transformation of natural substrates with low water solubility, such as puerarin. The biotransformation of natural products is usually conducted under mild conditions, rather than at an improper pH, high salt concentration, high substrate concentration, and high temperature. Thus, the use of immobilized enzymes in transformation of natural products effectively avoids detachment from the DEAE-52 cellulose carrier due to such improper reaction conditions (Bai *et al*., 2005; Karboune *et al*., 2005; Shi *et al*., 2010; Ashraf *et al*., 2010).

We have previously reported that puerarin is transformed to two products, puerarin-7-*O*-glucoside and puerarin-7-*O*-fructoside, when it is transformed by free enzyme or bacterial cells permeabilized with organic solvent (Jiang *et al*., 2008; Yu *et al*., 2010). However, we showed that the main transformation product by immobilized puerarin glycosidase is puerarin-7-*O*-glucoside. It is possible that DEAE-52 cellulose specifically adsorbs puerarin glucosidase under the experimental condition, indicating that immobilization of enzyme is a useful method to select the specific transformation products from multiple potential products. Furthermore, it is convenient to purify the transformation product by immobilized enzyme because of the lack of interference from crude cell extract.

Herein we show that the properties of puerarin glycosidase are improved after immobilization on DEAE-52 cellulose. (1) Immobilized enzyme exhibits improved pH stability at neutral and basic pH. (2) Immobilized enzyme has increased thermal stability and retains its activity from 30-40 °C; in contrast, the activity of free enzyme decreases quickly when the temperature deviates slightly from 40 °C. (3) Immobilized puerarin glycosidase retains 104.8% of the initial enzyme activity after storage at 4 °C for 45 days, while free enzyme retains 97.5% of the initial enzyme activity after storage under the same condition. To conclude, although the free puerarin glycosidase derived from *M. oxydans* CGMCC 1788 is quite stable, the immobilization process further improves the properties and stability of the enzyme, which is favorable for future industrial production of puerarin glycoside.

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