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Enzymatic transformation of 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) by immobilized α-cyclodextrin glucanotransferase from recombinant *Escherichia coli*

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ABSTRACT

This work aims to produce 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) from ascorbic acid and β-cyclodextrin with immobilized α-cyclodextrin glucanotransferase (α-CGTase) from recombinant *Escherichia coli*. Molecular sieve (SBA-15) was used as an adsorbent, and sodium alginate was used as a carrier, and glutaraldehyde (GA) was used as a cross-linker. The effects of several key variables on α-CGTase immobilization were examined, and optimal immobilization conditions were determined as the following: glutaraldehyde (GA, cross-linker) 0.01% (v/v), SBA-15 (adsorbent) 2 g/L, $CaCl_2$ 3 g/L, sodium alginate 20 g/L, adsorption time 3 h, and immobilization time 1 h. In comparison with free α-CGTase, immobilized α-CGTase had a similar optimal pH (5.5) and a higher optimal temperature (45 °C). The continuous production of AA-2G from ascorbic acid and β-cyclodextrin in the presence of immobilized α-CGTase was carried out, and the highest AA-2G production reached 21 g/L, which was 2-fold of that with free α-CGTase. The immobilization procedure developed here was efficient for α-CGTase immobilization, which was proved to be a prospective approach for the enzymatic production of AA-2G.

1. Introduction

Cyclodextrin glucanotransferase (EC 2.4.1.19, CGTase) is one of the important amylolytic enzymes and finds wide applications in the production of cyclodextrins (CDs) and α-1,4-linked cyclicol-igosaccharides [1]. CGTase can be used for the production of CDs via intra-molecular transglycosylation reaction, and also can be used for the production of functional substances like glycosylated ascorbic acid (AA) via intermolecular transglycosylation [2–4], in which the glycosyl residue is transferred from α-1,4-glucan or cyclodextrin to a suitable acceptor such as AA or sucrose [5].

Muto et al. found a type of glucosylated AA, namely, 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), which can be enzymatically synthesized via transglucosylation with CGTase [5,6]. AA-2G is extremely stable and nonreducible, and is considered to be superior to other chemically synthesized AA derivatives like AA 2-O-phosphate or AA 2-O-sulfate in terms of the reaction specificity and efficiency in large-scale production [7]. In addition to being stable in vitro and highly active in vivo, AA-2G also exhibits lipophilicity and may be used as components in cosmetics [8–10].

The enzymatic production of AA-2G is more applicable than chemical synthesis of the other AA derivatives due to the simple reaction steps, high regiospecificity and low production cost. The enzymatic transformation of AA-2G via the transglycosylation with CGTase is attracting an increasing interest. Markosyan et al. produced AA-2G with CGTase from mesophilic, thermophilic, and halophilic bacteria and maltase from the yeast *Saccharomyces cerevisiae* [11]. Jun et al. produced AA-2G via transglycosylation by CGTase from *Paenibacillus* sp. [12]. Currently, in most studies the CGTase used for the transformation of AA-2G is free, and there are few reports concerning the AA-2G production with the immobilized CGTase, though the latter has many advantages. Prousoontorn and Pantatan investigated the production of AA-2G with the immobilized CGTase from *Paenibacillus* sp. [2]. In their work, a covalent coupling method was used and the performance of the immobilized CGTase was evaluated. However, the immobilized CGTase cannot be used for the large scale production of AA-2G due to the low CGTase activity and AA-2G yield. Therefore, how to achieve

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the efficient CGTase immobilization is important for the enzymatic production of AA-2G.

CGTase has been immobilized on different supports by covalent binding, adsorption, entrapment or cross-linking [13–17]. A few reports have described the immobilization of CGTase covalently to supports, such as Eupergit C and glyoxyl-agarose [18–20], or by entrapment in sodium alginate beads [21,22], or by exchange with ionic bond [23].

Sodium alginate is a suitable carrier for entrapment immobilization of enzymes for industrial applications [24]. Glutaraldehyde activation of supports is one of the most popular techniques to immobilize enzymes. The glutaraldehyde is used to activate supports by physical adsorption and covalent binding, immobilizing the enzyme on a glutaraldehyde pre-activated support [25–27]. SBA-15 is porous silicates with huge surface areas (normally $\geq 500\,m^2/g$), large pore sizes ($2\,nm \leq size \leq 20\,nm$) and ordered arrays of cylindrical mesopores with very regular pore morphology. The large surface areas of these solids increase the probability that a reactant molecule in solution will come into contact with the catalyst surface and react. The large pore size and ordered pore morphology allow one to be sure that the reactant molecules are small enough to diffuse into the pores [28]. Chemical modifications of amino acids reduce any conformational change involved in enzyme inactivation and increase the enzyme stability.

In this work, the α-CGTase was immobilized with SBA-15 as an adsorbent, glutaraldehyde as a cross-linking agent, and sodium alginate as a carrier. Characterization of the immobilized enzyme for catalytic properties in comparison with the free enzyme was also investigated. The immobilized α-CGTase was used to repeat the production of AA-2G from ascorbic acid and β-cyclodextrin. It should be noted that here the developed α-CGTase immobilization is not a novel immobilization approach, but reaches a significant enhancement of AA-2G production via the combination of existing materials and technologies. This work may be helpful for the industrial production of AA-2G with the immobilized α-CGTase.

2. Materials and methods

2.1. Materials

Sodium alginate, glutaraldehyde (25% aqueous solution, GA), $CaCl_2$, β-cyclodextrin and ascorbic acid were purchased from Sangon (Shanghai, China). Molecular sieve (SBA-15) was purchased from Dragon Technology Co., Ltd. (Liaoning, China). AA-2G was purchased from Wako Pure Chemical (Wako, Japan). All other chemicals and reagents were of analytical grade.

2.2. Activity measurements

2.2.1. α-CGTase activity assay

The α-recombinant CGTase was assayed as the dextrinogenic activity by methyl orange method described in the literature [29]. The α-cyclodextrin forming activity was determined by the methyl orange method as described previously with some modifications. The culture or cytoplasmic supernatant (0.1 mL) (appropriately diluted in 50 mM sodium phosphate buffer) was incubated with 0.9 mL of 3% (w/v) soluble starch in 50 mM phosphate buffer (pH 6.0) at 40 °C for 10 min. The reaction was terminated by the addition of 1.0 M HCl (1.0 mL), and then 1.0 mL of 0.1 mM methyl orange in 50 mM phosphate buffer (pH 6.0) was added. After the reaction mixture was incubated at 16 °C for 20 min, the amount of α-cyclodextrin in the mixture was spectrophotometrically determined by measuring the absorbance at 505 nm. At the chosen conditions, the molar extinction coefficient of methyl orange was $\varepsilon = 2.98 \times 10^4\,L/(mol\,cm)$. One unit of α-CGTase activity was defined as the amount of enzyme that was able to produce 1 μmol of α-cyclodextrin per min under the above conditions.

2.2.2. Glucoamylase activity assay

One unit of glucoamylase activity is defined as the amount of enzyme which produces 1.0 mmol glucose from dextrin in 1.0 min at 50 °C at pH 5.5 [30].

2.3. Preparation of enzyme solution

The recombinant plasmid *cgt*/pET-20b(+), in which the *cgt* gene encoding the mature α-CGTase from *Paenibacillus macerans* strain JFB05-01(CCTCC M203062) was placed downstream of a DNA sequence coding pelB signal peptide, and *Escherichia coli* BL21(DE3) harboring plasmid *cgt*/pET-20b(+) were constructed previously [31].

The culture conditions for the extracellular production of CGTase from recombinant *E. coli* BL21 (DE3) in shaking flask were (g/L): glucose 8, lactose 0.5, peptone 12, yeast extract 24, K_2HPO_4 16.4, KH_2PO_4 2.3, and $CaCl_2$ 0.28. The initial pH was 7.0 and the culture temperature was maintained at 25 °C. The culture broth was centrifuged at $12{,}000 \times g$ at 4 °C for 5 min and the collected supernatant was hyperfiltrated.

2.4. Enzyme immobilization methods

2.4.1. Embedding of α-CGTase

Thoroughly mixed solution of 10 mL (160 U/mL) enzyme and 0.2 g sodium alginate was placed into 4 °C refrigerator overnight for degasification. The drops of the solution were added into $CaC1_2$ (3%) for 2 h hardening. The collected capsules were washed with distilled water until there was no protein precipitation. The washed capsules were stored in glycerol at 4 °C until subsequent use.

2.4.2. Crosslinking-embedding of α-CGTase

Ten milliliters (160 U/mL) of enzyme solution and 100 μL of glutaraldehyde (25%) were mixed completely and then cross-linked for 4 h. The mixture was added with 0.2 g sodium alginate, and then was placed in 4 °C refrigerator overnight for degasification. The drops of the solution were added into $CaC1_2$ (3%) for 2 h hardening. The collected capsules were washed with distilled water until there was no protein precipitation, and then the washed capsules were stored in glycerine at 4 °C until subsequent use.

2.4.3. Embedding-crosslinking of α-CGTase

The capsules collected via embedding method were washed with distilled water until there was no protein precipitation, and were further cross-linked for 4 h in 30 mL of glutaraldehyde solution (0.2%). The treated capsules were preserved in glycerol.

2.4.4. Adsorption-crosslinking-embedding of α-CGTase

The mixture consisting of 10 mL (160 U/mL) of enzyme solution and 0.01–0.1 g of molecular sieve was placed into a shaker at 16 °C and 150 rpm for 1–10 h adsorption, and then 100 μL of 25% glutaraldehyde was added for 4 h crosslinking. The solution was treated with 0.2 g sodium alginate and placed in 4 °C refrigerator overnight for degasification. The drops of the solution were added into $CaC1_2$ of 2–8% for 2 h hardening. The collected capsules were washed with distilled water until there was no protein precipitation, and then the washed capsules were stored in glycerine at 4 °C until subsequent use.

2.5. Enzymatic AA-2G production

L-Ascorbic acid and β-cyclodextrin were dissolved into 100 mM acetate buffer to yield final concentration of 5% (w/v) and 5%

(w/v), respectively. Five milliliters of the immobilized α-CGTase was added to 5 mL of substrates solution and incubated at 37 °C for 24 h with gentle orbital shaking (150 rpm) in the dark. Glucoamylase (10 U/mL) was added to the reaction medium and incubated at 65 °C and pH 5.5 for 6 h to hydrolyze the AA-2-oligosaccharides (AA-2Gs) to AA-2G. The activity of the immobilized α-CGTase was determined by assaying the rate of AA-2G production in the first 24 h.

2.6. Properties of immobilized α-CGTase

2.6.1. Effect of pH on the immobilized α-CGTase activity

The influence of pH on the activity of immobilized α-CGTase was studied by incubating mixture solutions in sodium acetate buffer with pH ranging from 4.1 to 6.4 and sodium phosphate buffer with pH ranging from 6.7 to 7.0. Other conditions are the same as described in Section 2.5.

2.6.2. Influence of temperature on the immobilized α-CGTase activity

The effect of temperature on the activities of free and immobilized α-CGTase was investigated by incubating mixture solutions at temperatures ranging from 30 to 60 °C.

Other conditions are the same as described in Section 2.5.

2.6.3. Reusability

The reusability tests were performed at 45 °C with a batch operation mode. Afterincubation, the immobilized enzyme was removed from the reaction medium, and was washed three times with citrate buffer (50 mM, pH 6.0) and then used again.

2.6.4. Operational stability

Operational stability of the immobilized CGTase was determined for AA-2G synthesis in a sealed plastic tube. L-Ascorbic acid and β-cyclodextrin were dissolved into 100 mM acetate buffer to yield final concentration of 5% (w/v) and 5% (w/v), respectively. The immobilized α-CGTase samples (1600 units) was added to 10 mL of the substrates solution and incubated in the reactor at 45 °C and 38 °C for 5 d with gentle orbital shaking (150 rpm) in the dark. Ten milliliters of free α-CGTase (160 U/mL) was added to 10 mL of the substrates solution and incubated at 38 °C for 5 d with gentle orbital shaking (150 rpm) in the dark. Samples were taken at regular time intervals (6 h) for the measurement of AA-2G with HPLC. The relationship between operation time and conversion was studied.

2.6.5. Storage stability

Immobilized α-CGTase was stored at 4 °C in 50 mM of citrate buffer. The storage stability of the free and immobilized enzyme was investigated by measuring their remaining activity once every 10 days.

2.7. AA-2G analysis

AA-2G was measured with HPLC equipped with a SB-Aq column (4.6 mm × 250 mm). Samples were previously filtered via a 0.45 μm membrane before injection. The assay conditions were as follows: the detection wavelength was 238 nm and the mobile phase was 0.025 M KH_2PO_4/H_3PO_4 (pH 2.0) at a flow rate of 0.5 mL/min. At these conditions, the standard AA-2G was separated reproducibly. The AA-2G concentration was calculated on the basis of peak area of standard curve.

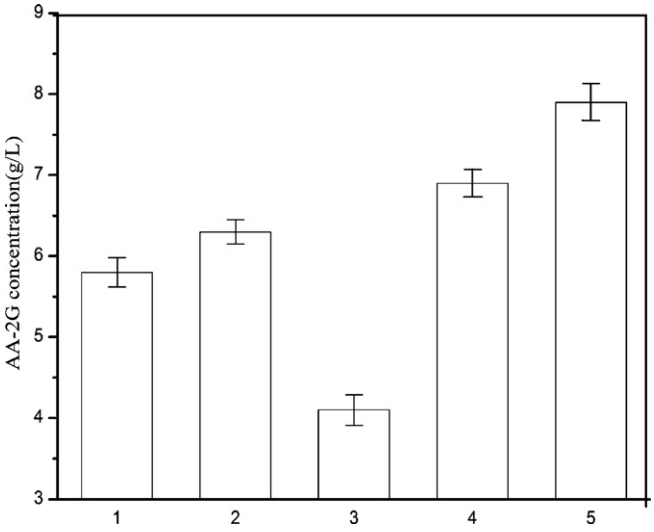

Fig. 1. Influences of immobilized enzyme and free enzyme on the synthesis of AA-2G. (1) Embedding of α-CGTase; (2) crosslinking-embedding of α-CGTase; (3) embedding-crosslinking of α-CGTase; (4) adsorption-crosslinking-embedding of α-CGTase; (5) free α-CGTase.

3. Results and discussion

3.1. Optimization of immobilization procedure

3.1.1. Immobilization methods

In spite of the fact that a large number of techniques and supports are now available for the immobilization of CGTase [2,19,32], there is not a universal means of immobilization [33] due to the fact that the choice of the support as well as the immobilization technique depends on the nature of the enzyme and the substrate, and its ultimate application. In this work, AA-2G concentration formed by free α-CGTase is 8.3 g/L (Fig. 1). Four different immobilization methods were developed, and the immobilized enzyme demonstrated high activity (>50%), in particular the adsorption-crosslinking-embedding immobilization showed the highest free activity of 90%. The high activity of adsorption-crosslinking-embedding immobilization may be attributed to the effective adsorption of free enzyme by SBA-15 as well as the stable covalent structure, which was formed by glutaraldehyde. Compared to the embedding method, crosslinking-embedding immobilization achieved a better result, indicating that enzyme crosslinked with glutaraldehyde was favorable for transformation. The worse was embedded-crosslinking immobilization, and the possible reason was that the gap formed by embedding was made so dense by glutaraldehyde crosslinking that it seriously hindered substrates to enter into to the active site of the enzyme, leading to a decrease in transformation efficiency.

3.1.2. The effect of SBA-15 concentration

Table 1 shows that SBA-15 is a molecular sieve with large aperture (average adsorption pore diameter 6 nm) and high specific surface area (≥500 m^2/g), which can enable fast adsorption

Table 1
SBA-15 performance indicators.

Index	Value
Surface area (m^2/g)	≥500
Pore volume (cm^3/g)	≥0.70
Cell wall (nm)	3–4
Average adsorption pore diameter (nm)	6

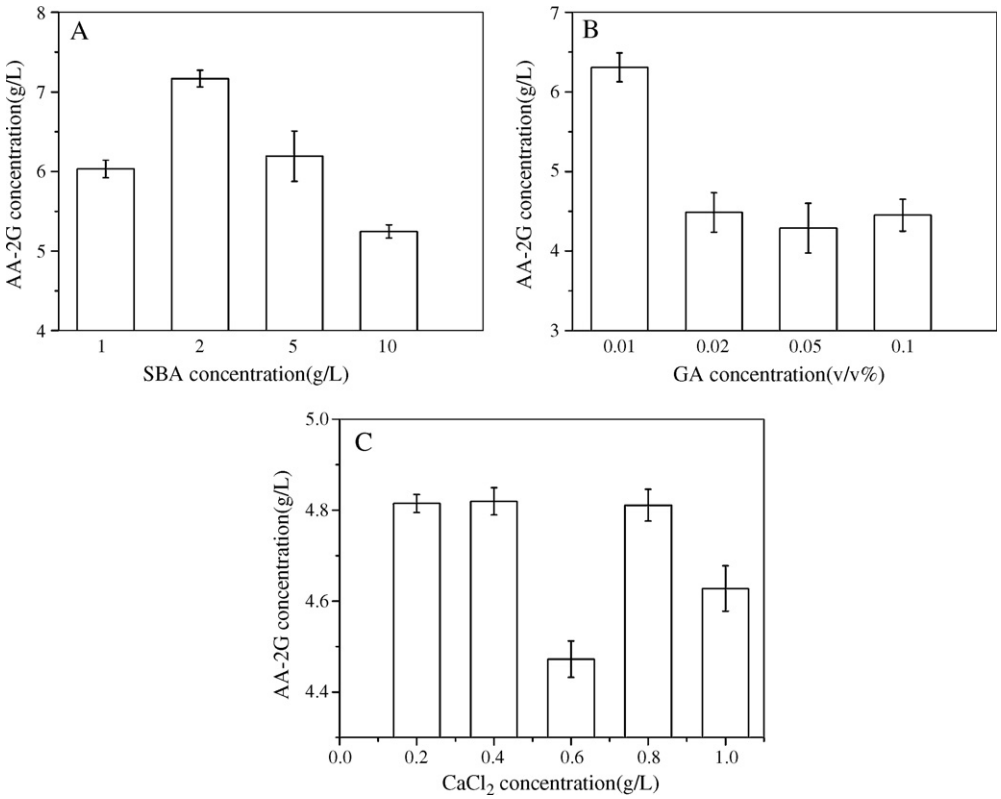

Fig. 2. Effects of SBA-15, GA and $CaCl_2$ concentrations on the conversion rate of the immobilized enzyme: (A) SBA-15, (B) GA and (C) $CaCl_2$.

of enzyme into the network holes. The optimal concentration of SBA-15 was found to be 2 g/L (Fig. 2A).

3.1.3. The optimum glutaraldehyde concentration

As a crosslinking agent, glutaraldehyde contains two aldehyde groups, which can form covalent bonds with enzyme, yielding three-dimensional crosslinked network structure. A low concentration (0.01%, v/v) of glutaraldehyde could enhance the catalytic reaction, however, when the glutaraldehyde concentration increased to 0.02%, it would damage the structure of the enzyme so that the conversion rate decreased (Fig. 2B).

3.1.4. Influence of $CaCl_2$ concentration

The Ca^{2+} concentration may affect the gel mesh size, thus affecting the entrance of substrates into the grid and the efficiency of enzyme. Therefore, different concentrations of $CaCl_2$ solution were used to examine its impact on immobilization of CGTase. Fig. 2C shows that the concentration of $CaC1_2$ has little effect on the conversion rate.

3.1.5. Adsorption time

The effect of adsorption time on the conversion of the immobilized enzyme has been studied with SBA-15 as the carrier (Table 1). As shown in Fig. 3A, the adsorption of enzyme on carrier increased initially only up to 3 h, and AA-2G concentration reached maximal at 3 h. It is possible that, part of the enzyme molecules could interact with the groups in the pore, causing the enzyme molecules to be retained at the orifice of the carrier. However, the remaining enzyme molecules were required to resist more blocking effect to enter the internal pore. With the extension of adsorption time, conversion rate decreased due to the reversibility of the adsorption and enzyme loss.

3.1.6. Calcification time

At the same conditions, changes in calcification time led to varied conversion rate. As shown in Fig. 3B, the optimal immobilization time is 1 h. Because Ca^{2+} played a role in protecting enzyme, 1 h of calcification time could achieve the optimal immobilization, but with the extension of time, the conversion rate was inhibited.

3.2. General properties of free and immobilized α-CGTase

3.2.1. Effect of pH

A comparative study between free and immobilized enzyme was provided in terms of pH of the medium. The pH is one of the most important factors influencing not only the dissociation of protein protons, but also the solution chemistry of the insoluble support [34–36]. Fig. 4 shows the effect of pH on the activity of free and immobilized α-CGTase. The optimum pH value of both free and immobilized enzyme was pH 5.0–5.5. This is an expected result, because the supports did not change the protein structure of immobilized enzyme acutely.

3.2.2. Influence of temperature

Fig. 5 shows the influence of temperature on the free and immobilized α-CGTase. The optimum temperature of free and immobilized enzyme were 38 °C and 45 °C, respectively. In comparison with the free CGTase, the immobilized CGTase had a higher optimal temperature and a broader temperature range of high activity.

3.2.3. Reusability

Reusability is of considerable importance for various applications of biocatalysts in a commercial point of view. The enzyme reuse means that the stability of the final enzyme preparation should be high enough to permit this reuse. Therefore, the enzyme needs to be very stable or to become highly stabilized during the

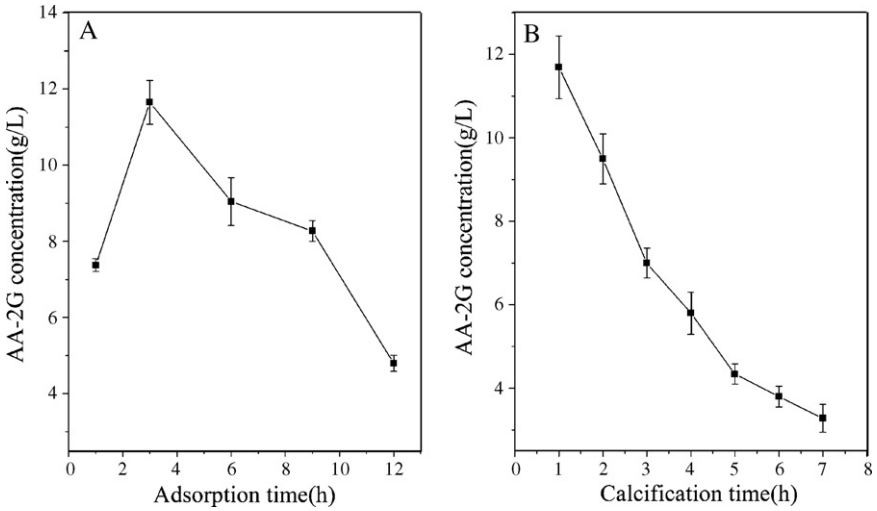

Fig. 3. Effects of (A) SBA-15 adsorption time and (B) immobilization time on the conversion rate of the immobilized enzyme: (□) free enzyme and (■) immobilized enzyme.

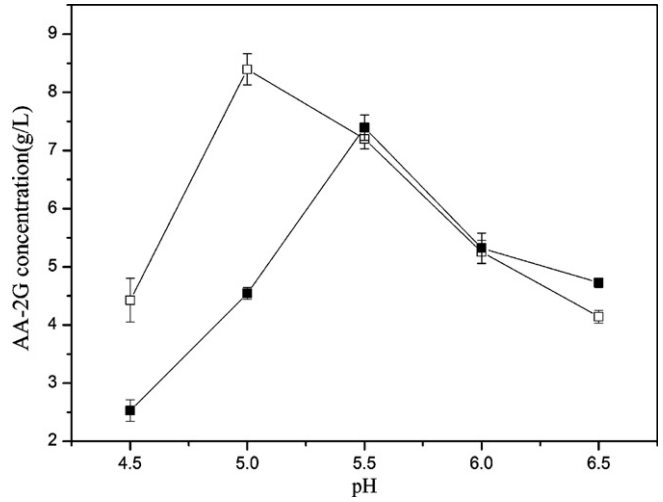

Fig. 4. Effect of temperature on immobilized and free α-CGTase activities: (□) free enzyme and (■) immobilized enzyme.

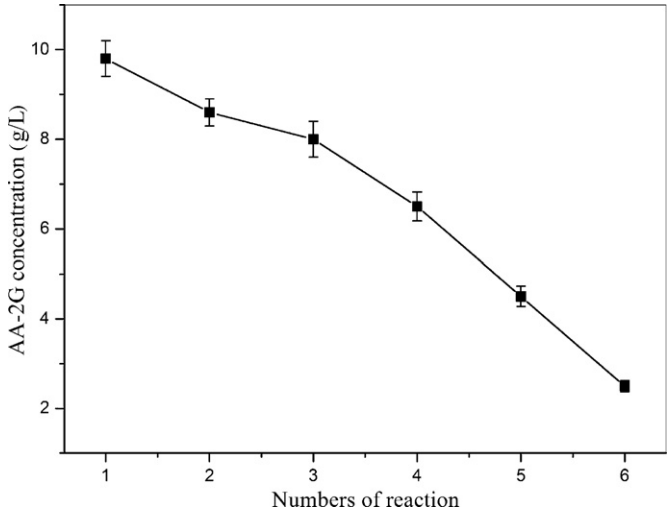

Fig. 6. Reusability of immobilized enzyme.

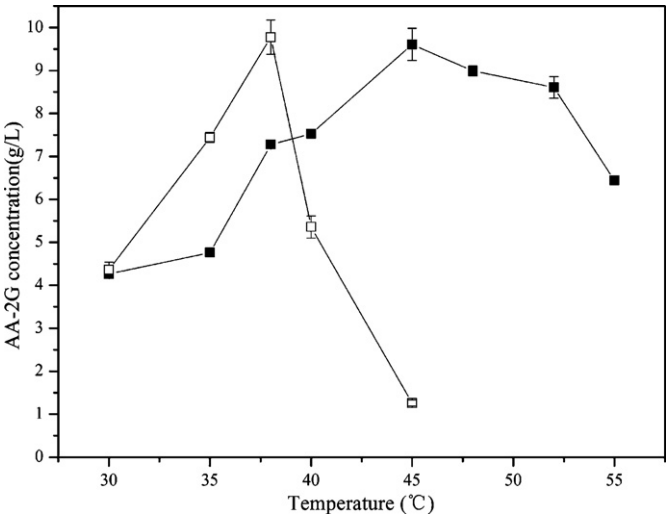

Fig. 5. Effect of pH on immobilized and free α-CGTase activities: (□) free enzyme (at 38 °C), (●) immobilized enzyme (at 38 °C) and (■) immobilized enzyme (at 45 °C).

immobilization process. The immobilized enzyme retained 65% of its initial activity after 4 cycles of reuse at 45 °C for 24 h (Fig. 6). This result showed that, the immobilized enzyme can be easily recovered and used repeatedly although significant loss of its activity. Prousoontorn and Pantatan reported that the immobilized CGTase retained its activity up to 74.4% of the initial catalytic activity after being used for 3 cycles [2]. The reusability of the immobilized CGTase in this work is a little lower in comparison with that reported by Prousoontorn and Pantatan [2], and this needs to be improved with further studies.

3.2.4. Operational stability

One of the most important parameters to be considered in enzyme immobilization was operational stability. Stability determines the possible application of enzyme in large scale processes. We produced AA-2G continuously using the immobilized α-CGTase in sealed plastic tube. The operation conditions were selected on the basis of the foregoing experimental results. Fig. 7 shows that the maximal concentration of AA-2G reached 9.6 g/L and 12 g/L at 38 °C in the presence of free α-CGTase and immobilized α-CGTase, respectively. At the optimal temperature 45 °C for the immobilized α-CGTase, AA-2G concentration reached 21 g/L, which was 2-fold of that in the presence of free α-CGTase. The conversion

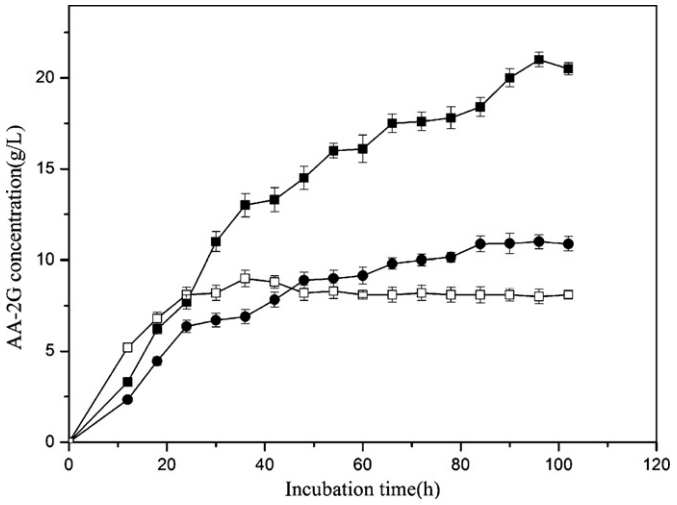

Fig. 7. The operational stability of immobilized enzyme: (□) free enzyme and (■) immobilized enzyme.

rate of the substrates with immobilized enzyme is 2-fold of that with the free α-CGTase. The molar yield and productivity of AA-2G were 0.22 (AA-2G/AA, mol/mol), 0.22 g L^{-1} h^{-1}, respectively. And for the immobilized CGTase in [2], the molar yield and productivity of AA-2G were 0.0073 (AA-2G/AA, mol/mol) and 0.01 g L^{-1} h^{-1}, respectively. The mole yield and productivity of immobilized α-CGTase on the synthesis of AA-2G was much higher than those of the immobilized α-CGTase from *Paenibacillus* sp. A11 [2].

3.2.5. Storage stability

The storage stability of the immobilized enzyme is an important parameter that determines the usefulness. Hence, the storage stability of the free and immobilized enzyme was investigated. The results revealed that the storage stability of the immobilized CGTase was improved compared with that of the free enzyme, and 4 °C was a suitable temperature for storage (Fig. 8). The immobilized a-CGTase retained 96% of its initial catalytic activity after stored at 4 °C for 2 months, and this is similar to what reported in [2], in which the alumina bound CGTase retained 96% of its initial catalytic activity when stored at 4 °C for more than 30 days.

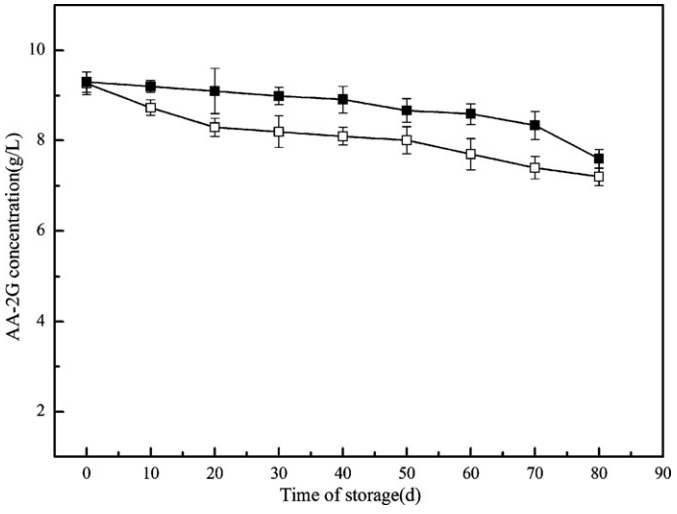

Fig. 8. Storage stability of immobilized enzyme: (□) free enzyme and (■) immobilized enzyme.

4. Conclusions

Immobilization of α-CGTase was conducted with SBA-15 as the adsorbent, glutaraldehyde as the cross-linking agent, and sodium alginate as the carrier. Optimal immobilization conditions were as following: GA 0.01% (v/v), SBA-15 2 g/L, $CaCl_2$ 3 g/L, adsorption time 3 h, and immobilization time 1 h. In comparison with the free α-CGTase, the immobilized α-CGTase had a similar optimum pH and a higher optimum temperature. The excellent properties of the immobilized enzyme are great for the production of AA-2G from ascorbic acid and β-cyclodextrin. At the optimal temperature, the conversion rate of the substrates with immobilized enzyme (45 °C) was 2-fold of that with the free α-CGTase (38 °C). The highest yield of AA-2G reached 21 g/L at 5 d with the immobilized α-CGTase. The immobilized α-CGTase retained up to 65% of its initial catalytic activity after being used for 4 cycles.

Acknowledgements

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