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Enzymatic conversion of Baicalin into Baicalein by β-glucuronidase encapsulated in biomimetic core-shell structured hybrid capsules

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ABSTRACT

In this study, Baicalein was produced through an enzymatic conversion catalyzed by β-glucuronidase (GUS) encapsulated in biomimetic alginate/protamine/silica (APSi) capsules. Experimental results indicated that the thermal and pH tolerance as well as the storage and recycling stability of GUS were significantly improved after encapsulation. Under the optimum conversion conditions (37 °C, pH 7), a high productivity of Baicalein (73%) was obtained. No loss in enzyme activity was observed after 11-day storage and 90% of the initial activity remained after 26-day storage. No appreciable loss in activity was found during 10 repeated reaction cycles. The facile encapsulation process, the high conversion efficiency and the enhanced stability set an encouraging example for converting natural compounds into high value-added functional products.

1. Introduction

Nowadays, conversion of natural raw materials into high value-added functional products has attracted considerable attentions in pharmaceutical and foods fields [1–5]. Conventional chemical conversion usually fails to meet the increasing requirements for drug and foods safety due to the involvement of basic or acidic catalysts and organic solvents. In comparison, enzymatic conversion shows promising applications owing to its unique advantages of non-toxicity, high efficiency, high selectivity and mild conversion conditions.

Baicalin, a major flavonoid derived from the root of *Scutellaria baicalensis* Georgi, has been proved to have anticancer, anti-HIV, antioxidant, antibacterial, and estrogenic efficacies. However, Baicalein, the aglycone of Baicalin, may merit much more attention since it exhibits higher activities in the above pharmacological functions [6–12]. In addition, Baicalein is more potent in bioavailability than Baicalin [13]. Unfortunately, however, the wide application of Baicalein is seriously limited by its low content (*ca.* 0.2%) in *S. baicalensis* Georgi. Enzymatic hydrolysis of Baicalin whose content is much higher up to 14% [14] should be a promising and feasible solution. Catalyzed by β-glucuronidase (GUS), the β-1,4-glucuronide bond of Baicalin can be cleaved to produce Baicalein efficiently (Fig. 1) [15].

For the large scale production of Baicalein, GUS is usually immobilized to enable reuse for a long period and thus significantly reduce cost. However, the GUS immobilized by the traditional covalent coupling [16,17] and sol–gel encapsulation [18] methods often suffers from low activity due to the substantial conformational change by the strong bonds involved in the covalent cross-linking or the harmful alcohols liberated from the sol–gel process. Therefore, efforts have been devoted to exploiting a "green" and facile method for GUS immobilization.

In our recent article [19], mimicking the existence state of GUS in natural environment, a carrier with a hollow capsule structure was designed and fabricated. Colloidal sodium carboxymethyl cellulose (CMC) was used as the "core" material, providing an enzyme-friendly microenvironment for the GUS inside. A rigid silica shell was constructed onto the soft Ca-alginate (Alg) membrane through a biomimetic silicification process inspired by protamine. It was extremely exciting to find that GUS encapsulated in such biomimetic alginate/protamine/silica (APSi) capsules exhibited fairly high activity and recycling stability.

As a continuance of the above work, the main objective of this study was to further discuss the formation mechanism of silica shell, extensively evaluate the stability of encapsulated GUS, screen

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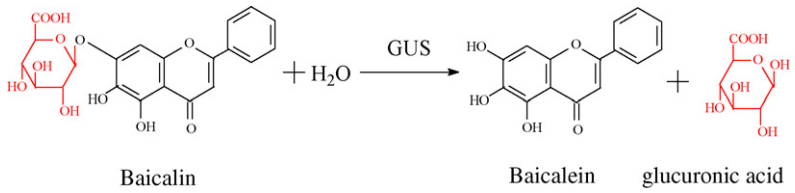

Fig. 1. Enzymatic conversion of Baicalin to Baicalein by GUS.

the optimum conversion conditions and assess Baicalein productivity of the enzymatic conversion.

2. Experimental

2.1. Materials

β-Glucuronidase from *Escherichia coli* (1,000,000–5,000,000 units/g protein) and protamine sulfate salt from salmon were purchased from Sigma Chemical Company. Sodium alginate (SA), CMC, and methyl cellulose (MC) were obtained from Tianjin Reagent Chemicals Co. Ltd. Sodium silicate (23% SiO_2) from Tianjin Jiangtian Chemical Co. Ltd. was used as silica precursor. Baicalin and Baicalein standards for analysis were obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China. Baicalin (purity ≥ 98%) used as substrate was purchased from Sichuan Xieli Pharmaceutical Co. Ltd. All the other chemicals were of analytical reagent grade.

2.2. Synthesis and characterization of silica precipitate

Protamine was suspended with a concentration of 5 mg/mL in pH 7.0, 30 mmol/L Tris–HCl buffer solution. MC and alginate were dissolved in buffer solution with a concentration of 1% (w/v). Freshly prepared 30 mmol/L sodium silicate solution was obtained by dissolving sodium silicate in water followed by acidification to pH 7.0 with HCl. Three kinds of organic macromolecules solution were mixed separately with sodium silicate solution in a ratio of 1:4. The mixture was allowed to react for 60 h. The resultant precipitate was collected by centrifugation, rinsed twice with deionized water and lyophilized to dryness.

The amount of silica in precipitate was confirmed by thermogravimetric (TG) analysis which performed in a PerkinElmer TG/DTA thermogravimetric analyzer by heating to 800 °C at a rate of 10 °C/min under air. The O/Si atomic ratio in precipitate was evaluated by EDS (Oxford) attached to the SEM (Philips XL30 ESEM).

2.3. Encapsulation of GUS in APSi capsules [19–22]

Tris–HCl buffer (30 mmol/L, pH 7) was used as a universal solvent to dissolve all the chemicals. CMC was dissolved in 0.10 mol/L $CaCl_2$ solution to give a final content of 2% (w/v). GUS was then added to the CMC–$CaCl_2$ solution to get an enzyme content of 0.1 mg/mL. 2 mL of the above CMC–$CaCl_2$–GUS solution was extruded through a 0.45 mm injection needle into 40 mL of stirred alginate solution (1.0% (w/v)). After 30 min, the mixture was diluted with 160 mL of deionized water before filtration. 10 mL of Tris–HCl buffer was used to remove the unreacted SA from capsule surface. The capsules were then transferred into 0.10 mol/L $CaCl_2$ solution to be further cross-linked for another 10 min. About 200 such Ca-alginate (Alg) capsules were recovered and thoroughly rinsed with Tris–HCl buffer twice. Subsequently, the capsules were placed in contact with 35 mL of 5 mg/mL protamine solution for 60 min. After filtration, capsules were added to 70 mL of 30 mmol/L sodium silicate solution (dissolved in water and acidified to pH 7 with HCl). The silicification reaction was allowed to proceed for 2 h. The capsules were removed, thoroughly rinsed with Tris–HCl buffer and stored at 4 °C.

2.4. Enzymatic conversion reaction and productivity

Equal amount of free or encapsulated GUS (*ca.* 0.14 mg) was introduced into a beaker containing 20 mL of 0.9 mmol/L Baicalin and 0.1% (w/v) Na_2SO_3, both dissolved in Tris–HCl buffer (30 mmol/L, pH 7). Na_2SO_3 was used here as an antioxidant. The beaker was tightly sealed and the reaction was performed at 37 °C under stirring. At different time intervals, the amounts of Baicalin and Baiclein in the reacting solution could be simultaneously detected by HPLC (HP1100, Agilent) equipped with Agilent ZORBAX SB-C18 column. A mixture of methanol:H_2O:H_3PO_4 (60:40:0.2) was employed as the mobile phase at a flow rate of 1 mL/min. The detection wavelength was set at 274 nm. Using the calibration curve of the Baicalin and Baicalein standards (data not shown), the concentration of Baicalin and Baicalein in the reaction solution could be calculated. Then, the amount of Baicalein produced and the unreacted Baicalin could be determined. The Baicalein productivity was defined as the mole ratio of the amount of Baicalein produced to the original amount of Baicalin in the feed. The enzyme activity unit was defined as the amount of GUS needed to produce 1.0 μmol of Baicalein per hour at 37 °C, pH 7.0.

2.5. Optimum temperature and pH

The optimum conditions for GUS activity were determined by changing the conversion temperature (30–70 °C) or pH (4–9), individually. The activity of GUS was calculated based on the amounts of Baicalein produced and was expressed as relative activity compared with the activity at optimum temperature and pH.

2.6. Thermal and pH stability

Thermal stability was investigated by introducing free and encapsulated GUS to Tris–HCl buffer solution followed by heating for 1 h at temperatures ranging from 30 °C to 70 °C. Then, the residual activity was measured at optimum temperature. Similarly, pH stability was evaluated by pre-incubating GUS in Tris–HCl buffer solution (pH 4–8) for 1 h and then performing the activity assay at optimum pH. The relative activity was calculated by taking the activity of GUS without incubation as 100%.

2.7. Storage stability

Free and encapsulated GUS were stored at 4 °C in 30 mmol/L Tris–HCl buffer solution (pH 7.0) for a certain period of time. The storage stability was compared by storage efficiency defined as the ratio of free or encapsulated enzyme activity after storage to their initial activity.

$$\text{Storage efficiency}\,(\%) = \frac{\text{enzyme activity after storage}}{\text{initial enzyme activity}} \times 100$$

Table 1
Silica precipitating ability of three kinds of organic macromolecules

Organic macromolecule	Control	Protamine	MC	Alginate
Functional groups of macromolecules	None	$=NH_2^+$	−OH	$-COO^-$, −OH
Electrostatic interaction between macromolecule and silicate	None	Attractive	None	Repulsive
Hydrogen bonding between macromolecule and silicate	None	+[a]	+	+
Time of appearance of precipitate (h)	48	0	0	7.5
Amount of precipitate (mg)	22.6	80.3	47.1	29.3
Amount of silica in precipitate (mg)	10.1	20.6	11.4	7.7
O/Si atom ratio in precipitate	1.73	1.93	1.90	0.84

[a] There exists hydrogen bonding interaction between macromolecule and silicate.

2.8. Recycling stability

The encapsulated GUS was filtered after each reaction cycle, rinsed three times with 10 mL of 30 mmol/L Tris–HCl buffer and immediately utilized in the next reaction cycle. The recycling stability of encapsulated GUS was evaluated by measuring the enzyme activity in each successive reaction cycle.

$$\text{Recycling efficiency}\,(\%) = \frac{\text{enzyme activity in the } n\text{th cycle}}{\text{enzyme activity in the 1st cycle}} \times 100$$

3. Results and discussion

3.1. Formation mechanism of silica shell on capsule

As indicated in our previous study [19], in order to improve the mechanical strength of Alg capsule and increase the recycling stability of encapsulated GUS, a silica shell was constructed on Alg capsule with the induction of protamine. In fact, when alginate/protamine capsules were immersed into neutral solution of sodium silicate, electrostatic interaction and hydrogen bonding could be simultaneously induced between the $=NH_2^+$ of protamine and the $Si–O^-$ of silicate. However, it was still not clear that which kind of interaction played a key role in protamine-templated silica precipitating. Moreover, alginate might also facilitate silica precipitating due to the hydrogen bonding between the −OH of alginate and the $Si–O^-$ of silicate. To clarify the above question and assumption, the silica precipitating ability of protamine, MC and alginate was examined and compared. As shown in Table 1, control experiment without any organic macromolecules showed no precipitates in the 30 mmol/L silicate solution within 48 h. In contrast, upon the addition of protamine, silicate solution became cloudy immediately, precipitation occurring due to the strong electrostatic attractive interaction and hydrogen bonding interaction between the protamine and silicate species. MC could also initiate precipitation immediately by hydrogen bonding interaction. When alginate was added to silicate solution, precipitate would be formed after 7.5 h of incubation via electrostatic repulsive interaction and hydrogen bonding interaction. These results suggested that the hydrogen bonding between organic macromolecules and silica precursor, by itself, could catalyze silica precipitating. Further analysis demonstrated that electrostatic attractive interaction helped to increase the amount of precipitate and the amount of silica in precipitate. On the contrary, electrostatic repulsive interaction could not only decrease the amount of silica in precipitate but also slowed down the silica precipitating rate.

Turning back to the silica formation process on alginate/protamine capsules, it could be deduced that hydrogen bonding between protamine and silicate accelerated silica formation, on the other hand, electrostatic attractive interaction allowed for the adsorption of silicate, thus increased the amount of silica on capsule surface. The combination of these two interactions resulted in the formation of intact silica shell. Furthermore, hydrogen bonding between alginate and silicate also made an additional contribution to the acceleration of silicification.

3.2. Thermal and pH stabilities of encapsulated GUS

GUS encapsulation was accomplished after the formation of silica shell on capsule. As shown in Fig. 2, encapsulation of GUS in APSi capsules led to an increase in both thermal stability and pH stability. Compared with the activity after being incubated at 30 °C, 92% of activity retained after being incubated at 50 °C for encapsulated GUS, whereas 57% of the free GUS activity retained after the same treatment (Fig. 2a). This enhanced thermal stability may be attributed to the protection function of both the hybrid silica shell and the liquid CMC core, where the latter one seemed to be more important since the enzymes were in direct contact with the liquid core. Some monosaccharides and polysaccharides have been reported to be able to improve the thermal stability of enzyme

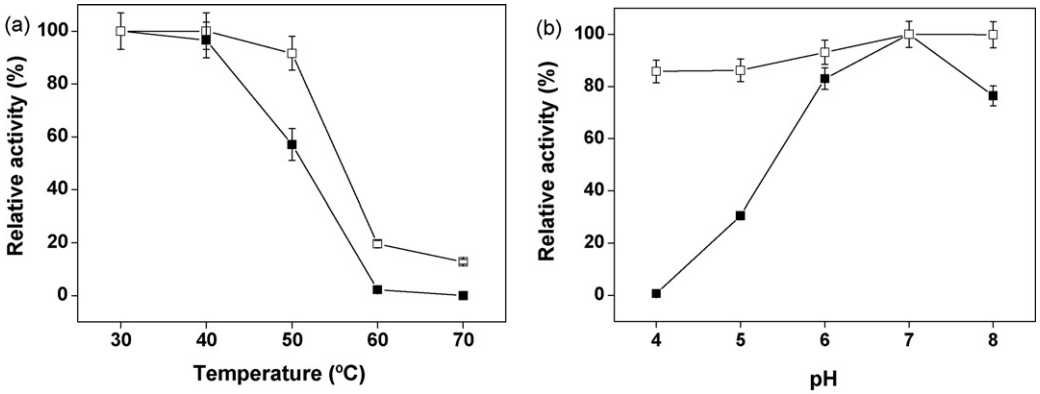

Fig. 2. Thermal (a) and pH (b) stability of free (■) and encapsulated GUS (□). (Each mean value was obtained by averaging five individual measurements. Error bars in the figures indicated the standard deviations of the mean value unless stated otherwise.).

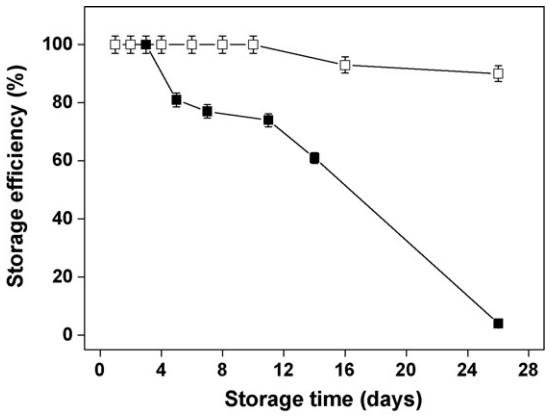

Fig. 3. Storage stability of free (■) and encapsulated GUS (□).

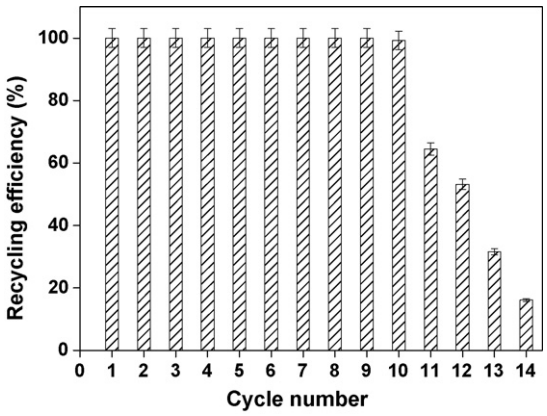

Fig. 5. Recycling stability of encapsulated GUS.

[23,24]. The fact that the denaturation temperature of free GUS was increased from 103 °C to 110 °C in the presence of CMC (data not shown) is a compelling evidence for the thermal stabilizing effect of CMC. The electrostatic repulsion between the negatively charged GUS and the also negatively charged CMC at neutral pH reduces the possibility of aggregation and conformational transition of GUS. On the other hand, the hydrogen bond interaction between the amino groups on GUS and the carboxyl groups on CMC contributes to the formation of a hydrophilic layer around GUS molecule [24] and strengthens the hydrophobic interactions among the nonpolar amino acids inside the GUS molecule [25], thus increasing the resistance of GUS to thermal inactivation. It was just the above two kinds of molecular confinement endowed CMC with the ability to maintain conformation of GUS under relatively high temperatures [26]. Incubation at a higher temperature of 60 °C led to a sharp decrease in activity, 20% of activity retained for encapsulated GUS and only 2% left for free one.

As shown in Fig. 2b, free GUS was markedly instable when incubated in acidic and alkaline medium, resulting in the non-reversible denaturation of GUS. The pH stability was significantly enhanced after encapsulation, the activity remained high in the whole range of pH 6–8. Especially, under the extremely acidic condition (pH 4), the relative activity of encapsulated GUS reached 86%, while its free counterpart lost activity almost entirely. This strong resistance of encapsulated GUS against the acidic and alkaline changes in medium was tentatively explained by the buffer function of the APSi capsule. In acidic medium, the negatively charged alginate shell and CMC core could attract and consume the H^+ ions to some extent, helping in preventing the H^+ from diffusing into and contacting with the enzymes. On the contrary, in alkaline medium, alginate shell and CMC core would release some H^+ ions, thus regulating the H^+ content inside the capsule. By this way, the pH change in the microenvironment in which the encapsulated GUS actually located is envisaged to be much smaller than that happened in the bulk solution.

3.3. Storage stability of encapsulated GUS

The storage stability of free and encapsulated GUS was compared in Fig. 3. The activity of free GUS decreased sharply by 20% after 5-day storage and continued falling down thereafter. On the 26th day of storage, only 4% of its initial activity was found. In contrast, the storage stability of GUS was significantly improved after encapsulation. No loss in activity was found during the first 10 days, and the remaining activity kept as high as 90% of its initial activity in 26 days. It was reasonably believed that the encapsulated GUS would exhibit a distinct advantage over free enzyme in Baicalin conversion after long-time storage.

The information on the possible change of enzyme conformation during storage in either Tris–HCl buffer or CMC solution was further clarified by CD study. The CD spectrum of freshly prepared GUS buffer solution displayed negative CD bands at 208 nm, 222 nm and 216 nm, representing α-helix and β-sheet structure, respectively. The solution became cloudy with the elapsing of storage time and the intensity of molar ellipticity ranging from 205 nm to 230 nm decreased. At the same time, a red shift of CD bands was also observed (Fig. 4a). These changes were indicators of denaturation which led to the sharp decrease in GUS activity, only 4% of its initial activity left after 26-day storage (Fig. 3). In comparison, the buffer solution of GUS in the presence of CMC remained as clear as

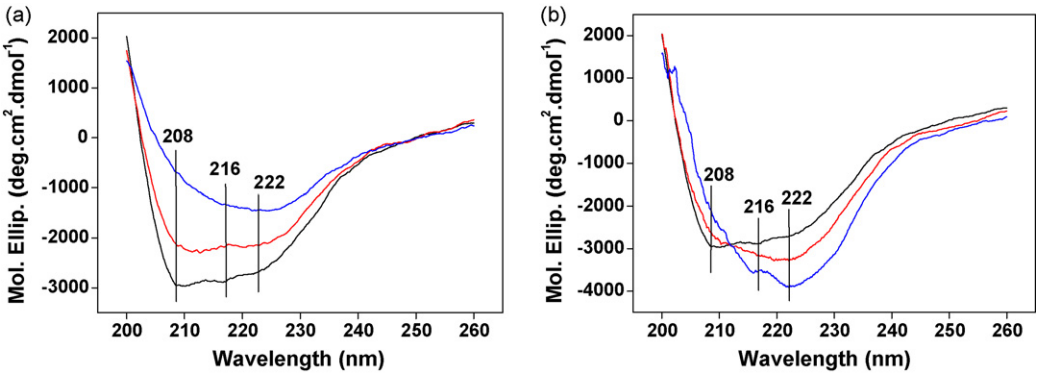

Fig. 4. CD spectra of GUS stored in (a) Tris–HCl buffer solution and (b) CMC solution. The GUS solutions were stored for 0-day (black line), 10 days (red line) and 26 days (blue line), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

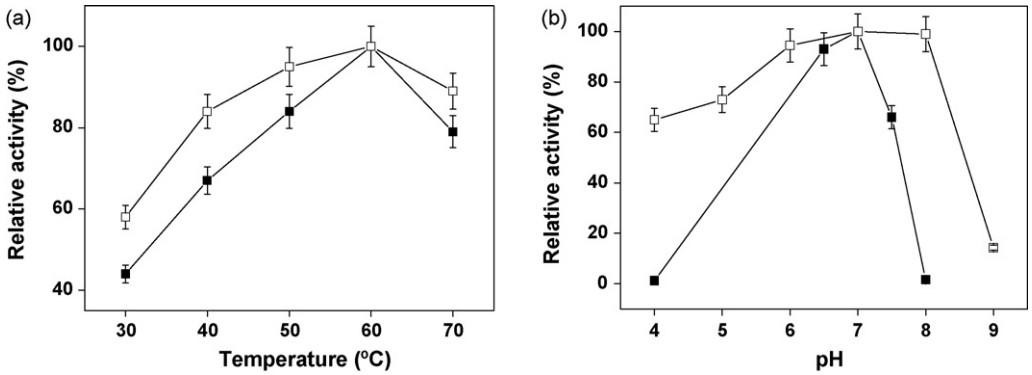

Fig. 6. Effects of (a) temperature and (b) pH value on the activity of free (■) and encapsulated GUS (□).

its initial state during the 26 days of storage. CD analysis revealed an interesting change in the secondary structure of GUS, that is, both the content of α-helix (222 nm) and that of β-sheet (216 nm) increased with the prolonging storage time (Fig. 4b). This increase might be due to the electrostatic repulsion and hydrogen bonding interactions between CMC and GUS. These unusual changes in conformation make a positive contribution to the enhanced storage stability of GUS encapsulated in the CMC core. In addition, the hybrid silica shell also helps in avoiding some unfavorable influences arising from the storage environment.

3.4. Recycling stability of encapsulated GUS

No appreciable loss in GUS activity was observed for encapsulated GUS after 10 repeated cycles (Fig. 5). The recycling stability of GUS encapsulated in APSi capsules was higher than that of enzyme encapsulated in Alg capsule [14] and Alg bead [27] prepared in our previous studies. It should be mentioned that the swelling degree of the above two kinds of carriers were larger than 100%. The swelling of APSi capsule was dramatically reduced by the formation of silica shell, and could be even completely inhibited by increasing the coverage rate of the silica shell [19]. Therefore, during the repeated conversion batches, the shape and the size of APSi capsules kept unchanged so that the pore structure of the capsule shell could be well maintained. Thus, the hybrid shell not only suppressed the leakage of encapsulated GUS, but also increased the mechanical strength of capsules. Consequently, the activity of encapsulated was notably stable even after multiple recycles under continuous stirring.

3.5. Optimum conditions for enzymatic conversion and Baicalein productivity

The encapsulated GUS was used to catalyze the hydrolysis of Baicalin. As shown in Fig. 6a and b, the highest activity for encapsulated GUS was achieved at 60 °C and pH 7, completely consistent with that of free GUS. It should also be noted that although the maximum activity was obtained at 60 °C, the thermal stability of GUS at this temperature seemed not sufficient for long-time reaction (Fig. 2a). In addition, Baicalin was reported to be unstable at temperature higher than 40 °C. Taking the effect of temperature on both activity and thermal stability into consideration, the optimum conditions for enzymatic conversion of Bacalin to Baicalein was determined at 37 °C and pH 7. The equilibrium productivity using free GUS reached 75% after 4 h reaction, while that using encapsulated GUS could reach a comparable level (73%) after a relatively longer reaction time (6 h) (Fig. 7). This high equilibrium productivity using encapsulated GUS was also comparable with that achieved by chemical conversion (*ca.* 80%) where inorganic

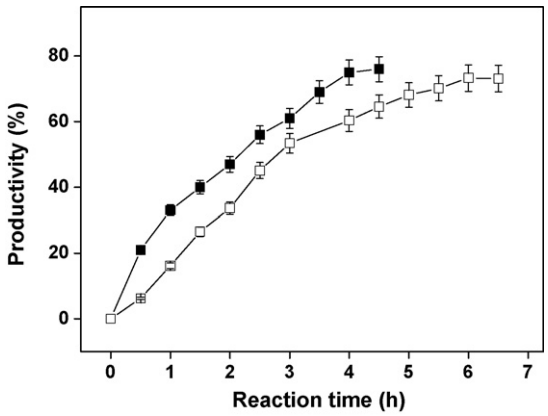

Fig. 7. Productivity of Baicalein with reaction time. (■) free GUS, (□) encapsulated GUS.

concentrated acids were used as catalysts [28]. Considering the practical application, enzymatic conversion by encapsulated GUS has its inherent advantages in terms of ease of separation and capability of recycling. Moreover, enzymatic conversion avoids the use of concentrated sulfuric acid and benzene which are not welcomed in pharmaceutical and foods industry.

4. Conclusions

In summary, GUS encapsulated in biomimetic hybrid APSi capsules displayed the following desirable properties for industrial application: (1) efficient Baicalin conversion and high Baicalein productivity of 73%; (2) enhanced stability towards temperature and pH changes; (3) most importantly, enhanced long-term storage and recycling stability. After 26 days of storage, the GUS using encapsulated GUS could still be maintained at 90%, while that in the case of free GUS was only 4%. In addition, no appreciable loss in activity was observed for encapsulated GUS after 10 repeated cycles. This study is expected to set an encouraging example for converting natural compounds into high value-added functional products.

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