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The immobilization of D-hydantoinase and characterization under classic condition and microwave irradiation

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Abstract

D-Hydantoinase was covalently immobilized onto polystyrene anion exchange resin via glutaraldehyde. Immobilization conditions were optimized: the carrier as D-92 type polystyrene anion exchange resin, temperature as 25 °C, immobilization time as 12 h, and initial concentration of protein as 6 mg/ml. Under the optimized reaction conditions the activity of the free and immobilized D-hydantoinase was determined. The free and immobilized D-hydantoinase samples were characterized with their kinetic parameters, thermal, and storage stability. The K_m and V_{max} values were 14.985 mM and 0.6 mM/min for the free, and 27.030 mM and 1.187 mM/min for the immobilized, respectively. Operational stability of the immobilized D-hydantoinase was also detected in a circulating packed-bed reactor. The half-time of the immobilized D-hydantoinase was 11 days. Nearly 90% of activity of the immobilized D-hydantoinase was reserved for 100 days stored at 4 °C. The free and immobilized D-hydantoinases were also characterized under microwave irradiation. Results shown that the reactions catalyzed by both free and immobilized D-hydantoinase were accelerated under microwave irradiation. The half-time of the immobilized D-hydantoinase reduced to 16 min under microwave irradiation.

Keywords: D-Hydantoinase; Immobilization; Polystyrene anion exchange resin; Microwave

1. Introduction

D-Amino acids are important medical, food, and pesticide intermediates, which can be widely used to produce some semisynthetic antibiotics, sweeteners, bioactive peptides, and chemical pesticides [1–3]. D-Phenylalanine, as an example, is used as a precursor for the synthesis of nateglinide, an antidiabetic agent [4].

D-Hydantoinase (EC 3.5.2.2) is one of the most useful enzymes in the D-amino acids production process. D-Amino acids can be produced from D,L-5-substituted hydantoins asymmetrically hydrolyzed by D-hydantoinase to *N*-carbamoyl-D-amino acids and these compounds are further converted to the corresponding D-amino acids by chemical method using sodium nitrite under acidic conditions or by a second enzymatic step using carbamoylase (EC 3.5.1.6) [5,6]. Because of the industrial significance, many studies have been performed for isolation and reconstruction of microorganisms containing D-hydantoinase and characterization of substrate specificity [7–9].

D-Hydantoinases producing from various microorganisms are obtained at present. However, free enzymes are too unstable to fulfill economical requirements for an industrially feasible biocatalyst. A few previous studies had reported immobilized D-hydantoinase or cells used to produce D-amino acids [10,11].

In view of high loading capacity, strong binding, low price, and especially transparent to microwave, polystyrene anion exchange resins were employed as a carrier for the immobilization of D-hydantoinase in the present work. We reported on results obtained during the optimization of the immobilization parameters for the D-hydantoinase crosslinked onto polystyrene anion exchange resins. The characterizations of immobilized D-hydantoinase under classical condition and microwave irradiation were investigated. Details are reported herein.

2. Materials and methods

2.1. Materials

D,L-Benzylhydantoin and standard *N*-carbamoyl-D-phenylalanine with eligible elemental analysis results were prepared from the corresponding amino acids [12]. *Burkholderia cepecia* JS-02 producing D-hydantoinase was

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screened from soil locally. All other reagents used were the analytical grade. MARS microwave reactor equipped with temperature-controlling optical fiber sensor was manufactured by CEM corporation, USA.

2.2. Culture of *B. cepecia* JS-02

The culture medium included (g/l): glucose 20, peptone 25, NaCl 3, KH_2PO_4 2, $MgSO_4$ 0.25, $CoCl_2$ 0.5, inducer 1.5, and pH was adjusted to 7.5 with 1 M NaOH. *B. cepecia* JS-02 was cultured in a 5 l mechanically stirred fermentor with 3 l culture medium at 32.5 °C, 550 rpm, and 0.8 vvm of air flow rate for 16 h. After cultivation, the culture was centrifuged at 6000 rpm at 4 °C for 15 min and precipitate cells were washed twice with pH 8.0, 50 mM Tris–HCl buffer.

2.3. Analytical methods

Protein concentration was measured by the method of Bradford. The assay of L- and D,L-benzylhydantoin, and *N*-carbamoyl-D-phenylalanine was performed on a Beckman PACE5000 HPCE instrument equipped with a UV detector. Separations were carried out at 25 °C on a 57 cm × 0.75 μm fused-silica column with 10 mM phosphate buffer, pH 8.5, and 25 kV. Wavelength detection at 200 nm was used to monitor the separations. Samples were injected under pressure for 5 s.

2.4. Preparation of crude D-hydantoinase solution

A 50 g of *B. cepecia* JS-02 cells suspended with 500 ml of pH 8.0, 50 mM Tris–HCl buffer (100 g l^{-1}) was lysed via ultrasonication under ice bath, and cell free lysate containing D-hydantoinase was prepared by centrifugation at 12,000 rpm for 20 min at 4 °C. Then the lysate was brought to 40% saturation with powdered ammonium sulfate, and allowed to stand for 2 h at 4 °C and centrifuged for 20 min at 12,000 rpm. The supernatant was collected. The procedure was repeated for ammonium sulfate saturation of 70%, and the precipitate was colleted. The fraction, which contained a large amount of D-hydantoinase was used for further purification by HIC (Pharmacia Phenyl Sepharose Fast Flow column). The column was equilibrated with 50 mM Tris–HCl buffer (pH 7.5) amended with 1 M $(NH_4)_2SO_4$. The column was eluted with 50 mM Tris–HCl (pH 7.5) at a rate of 2 ml min^{-1}, followed by 60%, 85%, and 100% step-wise $(NH_4)_2SO_4$ gradient in buffer. The fractions from the Phenyl Sepharose Fast Flow elution, containing the D-hydantoinase, were combined and dialyzed for 24 h at 4 °C. After that, the crude D-hydantoinase solution was refrigerated at 4 °C for further immobilization.

2.5. Immobilization of D-hydantoinase

Prior to immobilization, polystyrene anion exchange resins were treated with standard method [13]. Following the resins were treated with freshly 25% glutaraldehyde for 2 h at room temperature, then the remaining glutaraldehyde were removed thoroughly. Immobilization of D-hydantoinase was conducted at different temperature with constant shaking for hours. Unbound D-hydantoinase was decanted and loaded support was washed with distilled water prior to use and its storage at 4 °C.

2.6. Activity assay for free and immobilized D-hydantoinase

2.6.1. Free D-hydantoinase

Activity of free D-hydantoinase was determined by taken 2 ml of crude D-hydantoinase solution (protein concentration 8 mg/ml), 1.0 ml of substrate stock (2.0 g/l D,L-benzylhydantoin of 50 mM $NH_3{\cdot}H_2O$–NH_4Cl buffer, pH 8.5). The mixture was incubated with constant shaking at 38 °C for 10 min. Then the reaction was stopped by addition 0.25 ml of 10% trichloroacetic acid solution. The *N*-carbamoyl-D-phenylalanine was assayed described in Section 2.3.

2.6.2. Immobilized D-hydantoinase

Activity of immobilized D-hydantoinase was determined by taken 1 g of immobilized D-hydantoinase, 4.0 ml of substrate stock. The mixture was incubated with constant shaking at 38 °C for 10 min. Then the reaction solution was removed from the mixture. The resins were washed with 2 ml of assay buffer and the washings were also mixed with the reaction solution. The *N*-carbamoyl-D-phenylalanine was also assayed described in Section 2.3.

3. Results and discussion

3.1. The screening of immobilized supports

Weakly basic anion exchange resins were investigated in immobilization of D-hydantoinase. In all cases, the immobilization procedure was the same. Immobilization was committed by forming Schiff-base between free amino group in D-hydantoinase and aldehyde group in glutaraldehyde linker. The highest activity was obtained with D92-type prim-ammonium resin from Table 1. The higher activity of the D92-type resin

Table 1
Effect of various carriers on the activity of immobilized D-hydantoinase

Type	Amino group	Exchange capacity (mmol/g)	Residual activity (%)
D45	*prim-*	(a) ≥ 5.6	14
D80	*prim-*	(a) ≥ 6.5	16
D92	*prim-*	(a) ≥ 4.8	43.2
D82	*sec-*	(a) ≥ 3.5	20.1
DI5	*tert-*	(a) ≥ 6.5	11.5
D11	*tert-*	(a) ≥ 4.8	12
D01	*quarter-*	(a) ≥ 3.5	11
D01G	*quarter-*	(a) ≥ 4.0	15.3

Immobilization conditions: 1 g of support pretreated by glutaraldehyde and 10 ml of crude D-hydantoinase solution shaken at room temperature for 24 h. Then the resins were filtered and washed with the same $NH_3{\bullet}H_2O$–NH_4Cl buffer as described above. The activity of immobilized D-hydantoinase was determined according to the method in Section 2.6.2. Residual activity was defined as $U_{IDH}/(U_{FDH} - U_{SDH})$, here U_{IDH} represented the gross activity of immobilized D-hydantoinase, U_{FDH} represented the gross activity of free D-hydantoinase, and U_{SDH} represented the gross activity of supernatant D-hydantoinase.

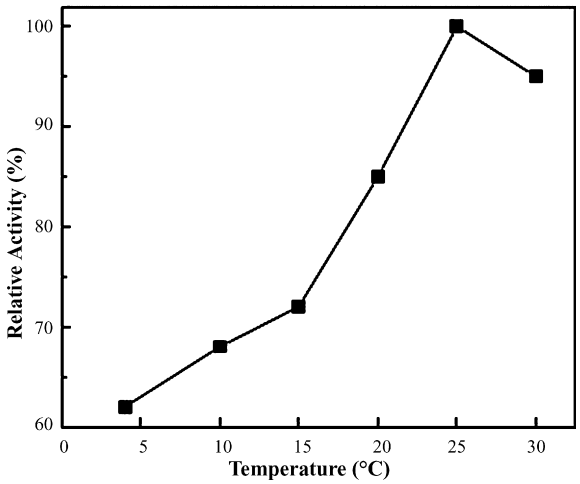

Fig. 1. Effect of temperature on immobilization of D-hydantoinase. One gram of support pretreated by glutaraldehyde and 5 ml of crude D-hydantoinase solution shaken at different temperature for 24 h, then removed the free enzyme completely to measure the activity.

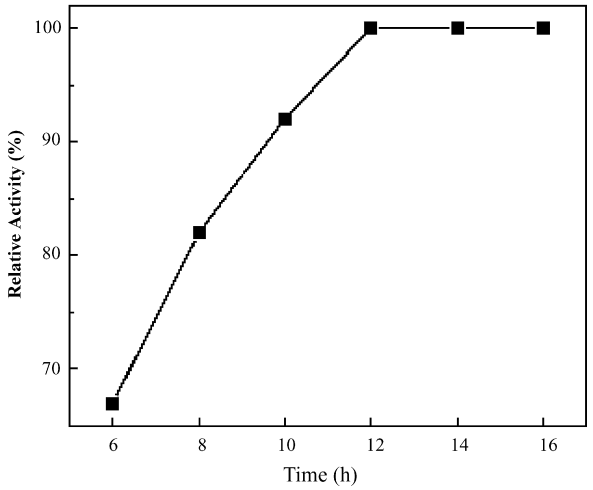

Fig. 2. Effect of period on immobilization of D-hydantoinase. One gram of support pretreated by glutaraldehyde and 5 ml of crude D-hydantoinase solution shaken at 25 °C for h then removed the free enzyme completely to measure the activity.

beads may be due to the difference in basicity compared with other resins. Alternatively, some conformational changes during the binding might also be responsible for the low activity [14]. Owing to a lack of sufficient structural information about the D-hydantoinase bound onto various supports, it is difficult to account for the low activity of the other supports.

Since the D92-type resin was the most promising of the supports tested, it was used for all further studies. It might be noted that the unloaded D92-type resin beads were not found to catalyze this test reaction.

3.2. Optimization of immobilization conditions

3.2.1. Effect of temperature on D-hydantoinase immobilization

As described in Section 2.5, D-hydantoinase was immobilized onto polystyrene resin bridged by glutaraldehyde to forming Schiff base. Temperature could be the most important factor to affect a chemical reaction rate to some extent. The immobilized effect was direct proportion to temperature at definite range. As shown in Fig. 1, the relative activity of immobilized D-hydantoinase was dramatically enhanced when the temperature increased from 5 to 25 °C. The immobilization temperature was maintained at 25 °C.

3.2.2. Effect of period on D-hydantoinase immobilization

The amount of enzyme adsorbed onto the support increased proportionally with time, leading to higher activity of preparations immobilized for longer durations. It was also found that the D-hydantoinase was immobilized during from 6 to 12 h showed certain residual activity, seen from Fig. 2. However, at and beyond 12 h, no significant increase in the activity of D-hydantoinase for the immobilized beads was found, indicating total adsorption of D-hydantoinase within 12 h. For all further experiments, therefore, the immobilization was carried out for 12 h.

3.2.3. Effect of protein concentration on D-hydantoinase immobilization

The activity displayed by the immobilized samples would be related to the amount of enzyme loaded onto it, and also its adsorption capacity. The effect of D-hydantoinase loading on the activity for the immobilized samples was shown from Fig. 3. The concentration of D-hydantoinase with up to 6 mg/ml, there was an enhancement in activity with the increase in amount of enzyme. Beyond this, an increase in the amount of D-hydantoinase produced no significant change in the activity displayed. The leveling-off of activity at higher enzyme loadings had also been found by Negishi et al. [15]. This phenomenon might be explained in terms of saturation of the enzyme molecule binding sites on the support.

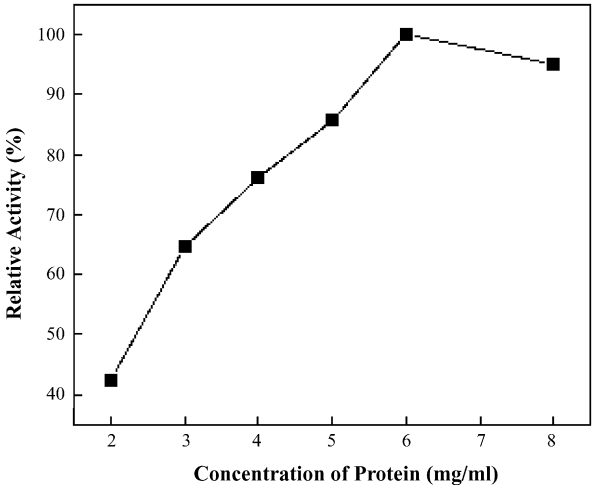

Fig. 3. Effect of concentration of protein on immobilization of D-hydantoinase. One gram of support pretreated by glutaraldehyde and 5 ml of crude D-hydantoinase solution with different protein concentration shaken at 25 °C for 12 h, then removed the free enzyme completely to measure the activity.

3.3. Characterization of free and immobilized D-hydantoinase

3.3.1. Effect of pH on activity

One of the most important parameters that affect the activity of enzyme is pH. This is more evident when the enzyme is immobilized since the support itself may change the pH value around the catalytic site. This effect is known as the partitioning effect, directly related to the chemical nature of the supporting material that induces electrostatic or hydrophobic interaction between the matrix and the molecular species dissolved in the solutions. These interactions alter the microenvironment in which the enzyme actually operates [16].

Effect of pH on the activities of free and immobilized D-hydantoinase were investigated and results were given in Fig. 4. It appeared that the optimum pH of free D-hydantoinase was 8.5, while it was shifted to 9.0 for the immobilized. We were expecting a considerable shift in optimal pH of immobilized D-hydantoinase to more basic region due to enabling solubility increase and racemization accelerating of L-enantiomer in substrate. However, the deviation in the optimal pH was less than that we expected. It might be explained that the immobilized D-hydantoinase was not chemically modified but remained as its native form loaded onto the resins. Similar results were also reported by Rai et al. [17] and Fan et al. [18].

3.3.2. Effect of temperature on activity

As we all know, high temperature is beneficial to chemical reaction, also to enzymatic reaction. It was very important to the reaction catalyzed by D-hydantoinase for increasing solubility and accelerating racemization of L-enantiomer in substrate when the reaction temperature was enhanced. As shown from Fig. 5, the optimum temperatures of free and immobilized D-hydantoinase were 38 and 45 °C, respectively. Difference in the optimum temperatures of immobilized D-hydantoinase was also reported by others [19]. It was obvious that optimal reaction temperature of D-hydantoinase was enhanced by immobilization. Even now, beyond 45 °C, the free and immobilized D-hydantoinase got inactivated dramatically, which might be explained that high temperature had changed the conformation of D-hydantoinase.

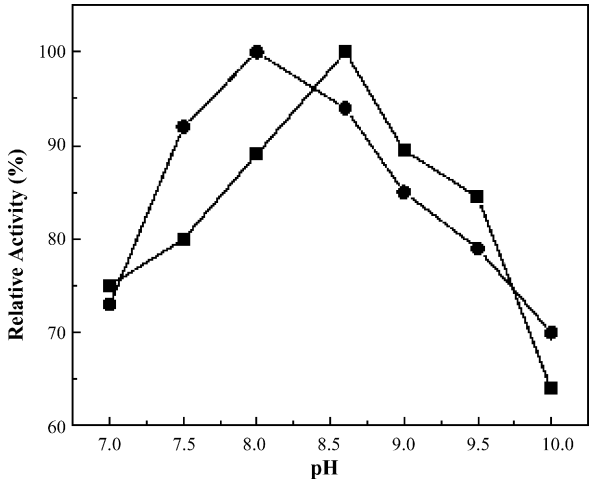

Fig. 4. Effect of pH on the activity of the free and immobilized D-hydantoinase. Activity of free and immobilized D-hydantoinase was determined at the same conditions at pH 7.0, 7.5. 8.0, 8.5, 9.0, 9.5 and 10. (●) Free D-hydantoinase and (■) immobilized D-hydantoinase.

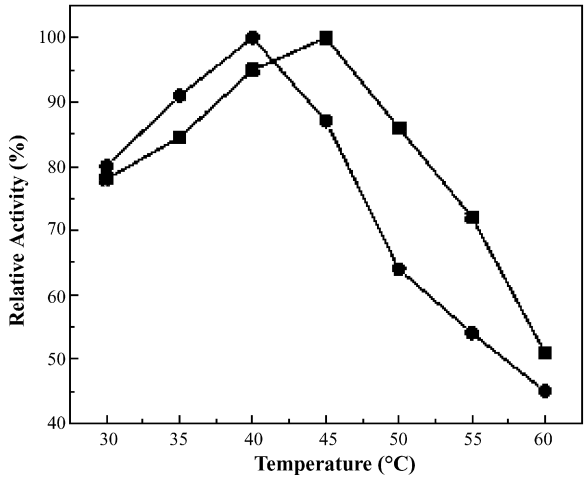

Fig. 5. Effect of temperature on the activity of the free and immobilized D-hydantoinase. The effect of temperature on the activity of free and immobilized D-hydantoinase was investigated for the temperature range of 30–60 °C at then optimal pH. (●) Free D-hydantoinase and (■) immobilized D-hydantoinase.

3.3.3. Effect of immobilization on kinetic constants

The effect of substrate concentration on the activities of the free and immobilized D-hydantoinase was investigated. Experiments were conducted at predetermined optimal conditions. Various benzylhydantoin concentration substrates were used to detect the primary velocity of free and immobilized D-hydantoinase at pH 8.5, 45 °C. The kinetic parameters of free and immobilized D-hydantoinase were calculated through Lineweaver–Burk plot. The K_m and V_{max} values were 14.985 mM and 0.6 mM/min for the free, and 27.030 mM and 1.187 mM/min for the immobilized, respectively. The both K_m and V_{max} values of the immobilized D-hydantoinase were almost two times higher than those of the free. The higher K_m values for the solid phase enzymes may be result of a number of effects. The migration of substrate from the solution to the microenvironment of an immobilized enzyme may be a major factor that causes an increase in K_m value [20]. In general, the main reasons were diffusion limitations and steric hindrances in the immobilized forms. It was apparent that it was not remarkable in our study.

3.3.4. Thermal stability of free and immobilized D-hydantoinase

The thermal stability of the enzymes was concerned deeply by the application in industry. To investigate the thermal stability of free and immobilized D-hydantoinase samples, experiments were carried out at eight different temperatures. It was observed that relative activity of the two forms was similar at temperature from 30 to 50 °C, shown in Fig. 6. At 60 °C, the immobilized D-hydantoinase remained 90 percent at least, but the free

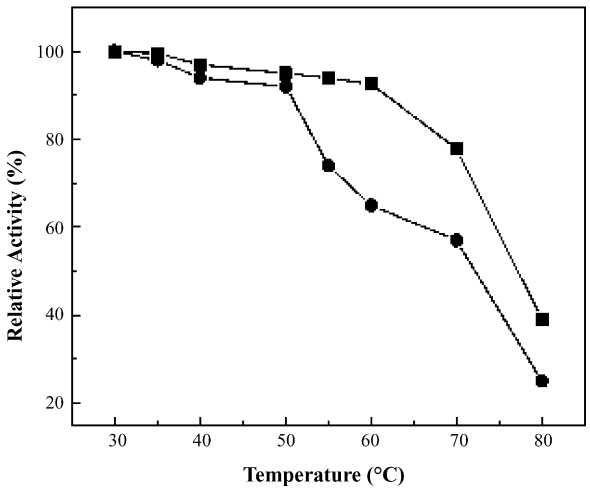

Fig. 6. Thermal stability of the free and immobilized D-hydantoinase. The preparations were first exposed to different temperatures for 30 min, then immediately cooled to 4 °C. The residual activity was detected at 38 °C (●) free D-hydantoinase and (■) immobilized D-hydantoinase.

form decreased sharply to 65% of the activity. So immobilization affected the thermostability of D-hydantoinase, yet not very notably.

3.3.5. Storage stability of free and immobilized D-hydantoinase

The storage stability of free and immobilized D-hydantoinase was detected. In our group, free D-hydantoinase remained the activity at least two weeks, reported by Li et al. [21]. In this study, the activity of immobilized D-hydantoinase was hardly decreased within 30 days, and nearly 90% of activity was reserved for 100 days. It was evident that immobilized D-hydantoinase was stable storage at 4 °C.

3.3.6. Operational stability of immobilized D-hydantoinase

Based on the results shown above, operational stability experiments of immobilized D-hydantoinase were carried out in a circulating packed-bed reactor. Concentration of D,L-benzylhydantoin solution was 4.0 g/l (50 mM $NH_3 \cdot H_2O$–NH_4Cl buffer, pH 8.5). The reaction was carried out continuously. Results shown that the half-time of the immobilized D-hydantoinase was 11 days.

3.4. Characterization of free and immobilized D-hydantoinase study under microwave irradiation

In organic synthesis, microwave irradiation can result in higher reaction rates and higher product yield compared with that of conventional heating. The study of microwave action on biological systems is an area of intense interest. Several studies had observed effects of microwave irradiation on enzymes in aqueous media that they had concluded to be nonthermal effect in origin [22–24]. Several papers had already shown that the kinetics of the enzyme-catalyzed reactions could be enhanced under microwave irradiation [25,26].

Table 2
The comparison of reaction catalyzed by free D-hydantoinase under microwave irradiation and classical condition

Temperature (°C)	Microwave		Classic		r_m/r_c
	r_m (mM/min)	Yield (%)	r_c (mM/min)	Yield (%)	
30	5.77	56.7	0.65	60.3	8.9
33	5.5	58.7	0.7	76.3	7.9
37	6.26	40.5	0.75	76	8.3
42	–	50	–	77	–

Five millilitres of 4 g/l D,L-benzylhydantoin solution and 2 ml of free D-hydantoinase was mixed starting reaction under classical condition and microwave irradiation. Reaction time: 1 min, microwave and 10 min, classical. Reaction rate: r_m microwave and r_c classical.

3.4.1. Effect of classical condition and microwave irradiation on the reaction rate and yield of free D-hydantoinase

The study of free D-hydantoinase under microwave irradiation was committed in a MARS microwave reactor. The results of the investigation were given from Table 2. The catalytic effect of free D-hyantoinase was higher under microwave irradiation than that of under classical condition at the same temperatures. A positive effect of microwave irradiation on the reaction catalyzed by D-hydantoinase was obtained. Although the yields of the products of the reaction for 1 min were in some short lower than that of under classical condition for 10 min, the former reaction rates were approximately eight times higher than that of the latter.

3.4.2. Effect of classical condition and microwave irradiation on the reaction rate and yield of immobilized D-hydantoinase

Resembling with free D-hydantoinase, immobilized D-hydantoinase was also tested, and the results were shown from Table 3. The yields of the products of the reaction for 1 min were a few higher than that of under classical condition for 10 min at 30 and 33 °C, and less than that of at 37 and 42 °C. But the reaction rate under microwave irradiation was about 30 times than that of under classical condition, obviously higher than that of free D-hydantoinase. The reason might be explained that the conformation of D-hydantoinase crosslinking onto resin was more stable under microwave irradiation than that of free D-hydantoinase.

Table 3
The comparison of reaction catalyzed by immobilized D-hydantoinase under microwave irradiation and classical condition

Temperature (°C)	Microwave		Classic		r_m/r_c
	r_m (mM/min)	Yield (%)	r_c (mM/min)	Yield (%)	
30	2.21	48	0.09	39.9	24.6
33	4.6	52	0.13	45	35.4
37	5.12	45.9	0.15	51	34.1
42	5.65	39	0.22	48	25.7

Five millilitres of 4 g/l D,L-benzylhydantoin solution and 1 g of immobilized D-hydantoinase was mixed starting reaction under classical and microwave irradiation. Reaction time: 1 min, microwave and 10 min, classical. Reaction rate: r_m, microwave, and r_c, classical.

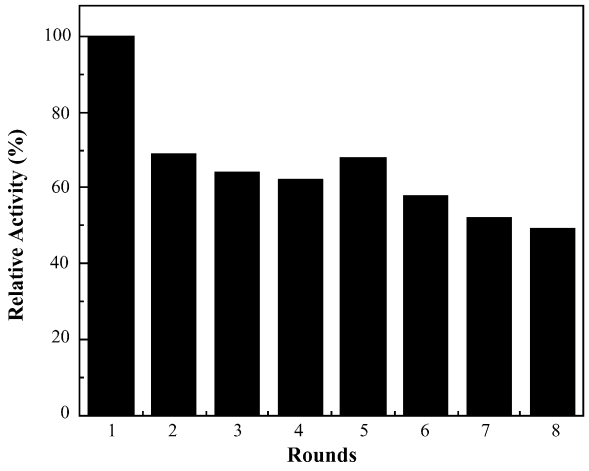

Fig. 7. Reusing experiments for immobilized D-hydantoinase under microwave irradiation. Batch experiments were conducted using 5 ml of 4 g/1 D,L-benzylhydantoin solution and 1 g of immobilized D-hydantoinase under microwave irradiation for 2 min. Then the reaction solution was removed thoroughly and the activity was detected.

According to this study, a conclusion could be drawn that reaction catalyzed by whether free or immobilized D-hydantoinase, microwave irradiation was a simple, effective method to promote reaction rate. The results were pretty consistent with that of reported by Lin, et al. [27] and Zarevúcka, et al. [28]. The reaction accelerated under microwave irradiation might be due to the sharp increase of probability of molecular collision.

3.4.3. Reusability of immobilized D-hydantoinase under microwave irradiation

The reusability of immobilized D-hydantoinase was tested under microwave irradiation, results shown in Fig. 7. Under microwave irradiation, the activity of immobilized D-hydantoinase would reduce to 50% in 16 min. Compared with 11 days of half-time under classical condition, the operation stability of immobilized D-hydantoinase under microwave irradiation was much less. Meanwhile, results showed that the immobilized D-hydantoinase lost the 30% of activity in initial 3 min. Under microwave irradiation, Lorentz forces produced from alternating electric field could destroy the activity center or three-dimensional structure of the enzyme. Therefore, the inactivation of the immobilized D-hydantoinase would be accelerated dramatically.

4. Conclusions

The parameters for the D-hydantoinase immobilized using polystyrene anion exchange resins were optimized. Biochemical characterization of the immobilized D-hydantoinase resulted in optimal pH and temperature. Compared with the optimal conditions of free D-hydantoinase, the immobilized shifted from 8.5 to 9.0 for the optimum pH, and from 38 to 45 °C for the optimum temperature. The reactions catalyzed by both free and immobilized D-hydantoinase were accelerated under microwave irradiation. Details about the mechanism should be probed by further experiments.

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