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Immobilization of glycolate oxidase from *Medicago falcata* on magnetic nanoparticles for application in biosynthesis of glyoxylic acid

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ABSTRACT

Glycolate oxidase was isolated from *Medicago falcata* Linn. after a screening from 13 kinds of C_3 plant leaves, with higher specific activity than the enzyme from spinach. The *M. falcata* glycolate oxidase (MFGO) was partially purified and then immobilized onto hydrothermally synthesized magnetic nanoparticles via physical adsorption. The magnetic nanoparticles were characterized with scanning electron microscope (SEM), transmission electron microscopy (TEM) and Fourier transform infrared (FT-IR) spectroscopy. The maximum load of MFGO was 56 mg/g support and the activity recovery was 45%. Immobilization of MFGO onto magnetic nanoparticles enhanced the enzyme stability, and the optimum temperature was significantly increased from 15 °C to 30 °C. The immobilized biocatalyst was successfully used in a batch reactor for repeated oxidization of glycolic acid to synthesize glyoxylic acid, retaining *ca.* 70% of its initial activity after 4 cycles of reaction at 30 °C for nearly 70 h, and its half-life was calculated to be 117 h.

1. Introduction

Glycolate oxidase (GO, EC. 1.1.3.15) is a peroxisomal enzyme which catalyzes the oxidation of α-hydroxy acid. It widely exists in the green parts of high plants to catalyze the second reaction of the photorespiratory pathway, *i.e.*, the oxidation of glycolate to yield glyoxylate and H_2O_2 [1–3]. GOs also exist in many animals' liver to form oxalate by converting glycolate to glyoxylate, the precursor of oxalate [4,5]. Among various GO sources, the spinach GO has been purified and characterized, and its crystal structure has also been dissolved correctly [6–8].

The application of spinach GO in the biocatalytic production of glyoxylic acid was intensively studied [9–11]. By using soluble spinach glycolate oxidase [9] or engineeried recombinant microbial whole cells [10] as biocatalysts, glycolic acid of 0.25–1.5 M was transformed into glyoxylic acid with high selectivity (>98%) at greater than 99% conversion. The enzymatic oxidation of glycolic acid was accompanied with the formation of by-product H_2O_2. The glyoxylic acid formed would be decomposed by H_2O_2 into formic acid and carbon dioxide or be further oxidized into oxalic acid by GO and oxygen. The by-product H_2O_2 could be decomposed by catalase and the further oxidation of glyoxylic acid could be prevented by the addition of ethylenediamine (EDA), which could react with glyoxylic acid to form *N*-substituted hemiaminal or imine [9]. The application of microbial GO for the production of glyoxylic acid was also reported [11], yielding 0.95 M glyoxylic acid after 15 days of transformation.

Soluble GO was unstable in aqueous medium and could not be reused, therefore the immobilization of GO was investigated. The spinach GO had been immobilized onto various supports, including oxirane acrylic beads [12,13] and silica sol–gel [14]. In the work of Anton et al. [12,13], the spinach GO was coimmobilized with catalase on Eupergit® C, an oxirane acrylic beads, resulting in an increased stability and prolonged operation time of the enzyme, but the activity recovery was only 22%, which was relatively low. The spinach GO was also immobilized on silica sol–gel [14], but a 70% activity loss was observed unless the GO was complexed with poly(ethyleneimine) prior to immobilization. So it was important to find an ideal support for the immobilization of GO with high activity recovery and stability.

Recently the researches on magnetic nanoparticles are of great interest [15–19] and the use of magnetic beads for immobilizing enzymes has been widely investigated [20–24]. Magnetic bead is used as a carrier of biomolecules because of the following advantaged properties [25]: (1) its diameter is much smaller than other supports, such as Eupergit® C, which can cause the perfect dispersal and great delivery of substrate molecules; (2) its high specific surface area can greatly enhance the protein adsorption capacity; and (3) it can be separated easily from the reaction mixture by applying a magnetic field. The advantage in recovery of enzyme from reaction system can also meet the requirements of industrial application. A variety of methods have been employed for immobilizing enzyme onto magnetic beads, such as cross-linking [26] and covalent attachment [27], however none of these reports has ever mentioned the

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immobilization of enzyme onto magnetic beads by simple physical adsorption.

Till now, no information was available regarding the immobilization of GO onto magnetic nanoparticles. In this work, the glycolate oxidation activity was examined among C_3 plants, which had been well known of possessing more GOs than C_4 plants [28], and a facile way was developed to immobilize the GO from *Medicago falcata* Linn. (abbreviated as MFGO) onto magnetic nanoparticles via a simple and mild way of physical adsorption. The reuse of the immobilized MFGO in the biotransformation of glyoxylic acid was investigated.

2. Experimental

2.1. Materials

Fresh plants were bought from market nearby. Bovine serum albumin (BSA) was purchased from Sigma–Aldrich Chemical Co. Ltd. Glycolic acid (70%, w/v), glyoxylic acid (50%, w/v) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) were purchased from Alfa Aesar. Flavin mononucleotide (FMN) was obtained from AppliChem GmbH. Phenylhydrazine hydrochloride and all other chemicals were supplied by Sinopharm Chemical Reagent Co. Ltd. Ethylenediamine (EDA) was obtained from Shanghai Lingfeng Chemical Reagent Co. Ltd.

2.2. Enzyme screening among C_3 plants

Different kinds of C_3 plants were selected as the source of glycolate oxidase. The fresh leaves (5 g) of these plants were triturated with 7 ml of potassium phosphate buffer (100 mM, pH 8.0) at 4 °C, and then centrifuged at 1000 × *g* for 5 min. The cell free extracts were collected, and the total amount of protein and the glycolate oxidation activity were measured.

2.3. Partial purification of glycolate oxidase from M. falcata Linn. and catalase from Baker's yeast

Glycolate oxidase from *M. falcata* Linn. (MFGO) was partially purified using selective ammonium fractionation. Fresh leaves (100 g) were chopped into fine particles using a tissue disruptor in 140 ml phosphate buffer (100 mM, pH 8.0). The liquid fraction was filtrated by 4 layers of gauze, then centrifuged at 6000 × *g* for 8 min, and the supernatant (*approx.* 0.2 l) was collected for ammonium fractionation.

Solid ammonium sulfate (9.35 g) was added into the cell free extract slowly. After all the ammonium sulfate was dissolved, the resulting precipitate was removed by centrifuge at 17,000 × *g* for 8 min, the pellet was discarded and then another 9.35 g solid ammonium sulfate was added into the supernatant (*approx.* 0.17 l) as before. All the steps above were carried out at 4 °C. The pellet was collected by centrifuge, lyophilized and stored at 4 °C until use.

Fresh cells of Baker's yeast were suspended in Tris–HCl buffer (20 mM, pH 7.5) and disrupted by high-pressure homogenizer AH110B (ATS Engineering Inc., Italy). The liquid fraction was centrifuged at 20,000 × *g* for 8 min and then the supernatant was collected for ammonium fractionation. The ammonium sulfate saturation was ranged from 40% to 60%, and the pellet was collected by centrifugation. All the steps above were carried out at 4 °C. The resultant pellet was dissolved in a small quantity of Tris–HCl buffer and dialyzed against the same buffer. After being lyophilized, the catalase was stored at 4 °C until use.

2.4. Preparation of amine-functionalized magnetic nanoparticles

The amine-functionalized magnetic nanoparticles used in the immobilization of enzyme were prepared by hydrothermal synthesis [29]. In brief, a solution consisted of 1,6-hexanediamine (3.6 g), anhydrous sodium acetate (4.0 g) and $FeCl_3{\cdot}6H_2O$ (1.0 g) as a ferric source in glycol (30 ml) was stirred to form a transparent solution, then transferred into a Teflon-lined autoclave and reacted at 200 °C for 6 h. The particles formed were rinsed with hot water and ethanol in turn to remove the solvent and unbound 1,6-hexanediamine, and then dried at 50 °C. The amine-functionalized magnetic nanoparticles were characterized by Fourier transform infrared (FT-IR) spectroscopy; its size and morphology were observed by scanning electron microscopy (SEM).

2.5. Adsorption of glycolate oxidase onto magnetic nanoparticles

The partially purified glycolate oxidase from *M. falcata* Linn. (MFGO) was immobilized onto amine-functioned magnetic nanoparticles via physical adsorption. The magnetic beads (0.5 g) were resuspended in 25 ml Tris–HCl buffer (100 mM, pH 9.0, containing 0.01 mM FMN) by ultrasonication, and then the MFGO was added, with a final concentration ranged from 10 mg/ml to 60 mg/ml. The immobilization process was carried out at 15 °C with a shaking rate of 160 rpm for 24 h. The immobilized enzyme was collected by magnetic field, washed 4 times with fresh buffer and then stored at 4 °C in the same buffer until use. Its morphology was observed by transmission electron microscopy (TEM). The amounts of protein remained in the enzyme solution before and after immobilization were determined by Bradford protein assay method.

2.6. Assay of enzyme activity

Activities of glycolate oxidases in different C_3 plants during screening were assayed spectrophotometrically at 324 nm by monitoring the formation of phenylhydrazone using a literature method [30] with slight modification. The phenylhydrazine can be converted to phenylhydrazone in the presence of glyoxylate at 30 °C and the phenylhydrazone formed has a maximum adsorption at 324 nm. The assay reaction mixture contained 2.5 ml phosphate buffer (100 mM, pH 8.0), 0.3 ml phenylhydrazine hydrochloride solution (100 mM, pH 8.0), 0.1 ml glycolate (100 mM, pH 8.0) and 0.1 ml enzyme solution. The enzymatic reaction was initiated by the addition of glycolate and the increase of absorbance at 324 nm was recorded every 30 s interval. One unit of glycolate oxidase activity is defined as the amount of enzyme that catalyzes the formation of 1.0 μmol glyoxylic acid per minute at pH 8.0 and 30 °C.

Catalase activity was assayed by measuring the change in concentration of discomposed H_2O_2 at 240 nm. The reaction was initiated by the addition of 0.1 ml catalase solution to 2.9 ml H_2O_2 solution (20 mM, pH 7.5), and the increase of absorbance at 240 nm was recorded every 30-s interval. One unit of catalase activity is defined as the amount of enzyme that catalyzes the decomposition of 1.0 μmol H_2O_2 per minute at pH 7.5 and 30 °C.

During the investigation of MFGO immobilization, the activities of the free and immobilized GOs were assayed using MBTH method which was first reported by Paz et al. [31] with slight modification. The free or immobilized GO was added into 2 ml Tris–HCl buffer (100 mM, pH 9.0) containing 50 mM glycolic acid and 0.01 mM FMN, the reaction was carried out at 30 °C for 30 min with a shaking rate of 160 rpm, then stopped with the addition of 0.4 ml HCl solution (1.2 M). After centrifuged, the glyoxylic acid formed in the reaction medium was determined with the addition of 0.2 ml MBTH (1%) and 2.5 ml $FeCl_3$ (0.2%) solution. The amount of tetraazopentamethine cyanine dye formed by glyoxylate, MBTH and Fe^{3+} was measured spectrophotometrically at 610 nm. One unit of glycolate oxidase activity is defined as the amount of enzyme that catalyzes the conversion of 1.0 μmol of glycolic acid to glyoxylic acid per minute at pH 9.0 and 30 °C.

2.7. Effects of pH and temperature on the activity of free and immobilized GOs

Effects of pH and temperature on the initial reaction rate and the stability of free and immobilized GOs were determined under various pHs (ranging from 6.0 to 11.0) and temperatures (ranging from 4 °C to 50 °C) by using MBTH method described above.

2.8. Determination of kinetic parameters of free and immobilized GOs

The apparent kinetics parameters K_m and V_{max} of the free and immobilized GOs were determined at 15 °C and 30 °C respectively, by measuring the initial reaction rate using glycolic acid as substrate by MBTH method. The K_m and V_{max} were calculated from Lineweaver–Burk plots, the formula was as follows:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{C_S} + \frac{1}{V_{max}} \quad (1)$$

where V is the initial rate of reaction at different substrate concentrations (C_S), K_m is the Michaelis constant, and V_{max} is the maximum reaction rate.

2.9. Repeated reaction in batch reactor using immobilized MFGO

Immobilized MFGO was used for repeated transformation of glycolic acid in a batch stirred reactor at a controlled temperature. The reaction mixture (50 ml) was composed of 100 mM glycolic acid, 0.01 mM FMN, 102 mM EDA and 2.0×10^5 U of catalase (partially purified from Baker's yeast); the reaction was initiated by the addition of 500 mg immobilized GO with an initial activity of 214 U/g supports at 30 °C and pH 9.0. The immobilized MFGO was separated from the reaction mixture by a magnet after each batch of reaction and washed with 200 ml Tris–HCl buffer (100 mM, pH 9.0, containing 0.01 mM FMN), and then added into the fresh reaction medium for the next cycle of reaction. The reaction was repeated for 4 times to evaluate the operational stability of the immobilized biocatalyst.

The concentration of glyoxylic acid produced was monitored by the MBTH method. The by-products formic acid and oxalic acid were identified and the amounts were measured by ion chromatography (IC) using ICS-1500 Ion Chromatography System with an analytical column of Ion Pac AS11-HC (Ø 4 mm × 250 mm), eluted by 30 mM NaOH solution with a flow rate of 1.0 ml/min at 30 °C. The retention times for formic acid, glyoxylic acid and oxalic acid were 3.15 min, 3.52 min and 4.23 min, respectively.

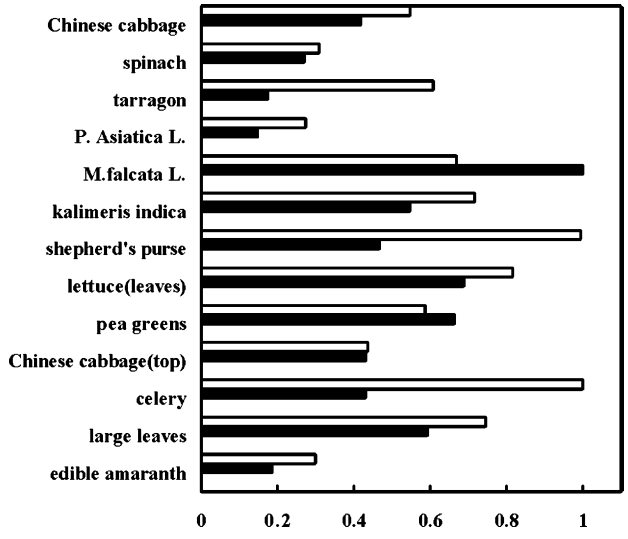

Fig. 1. Total yield (white bar, IU/g wet plant leaves) and specific activity (black bar, IU/mg protein) of glycolate oxidase extracted from different plant sources.

3. Results and discussion

3.1. Enzyme sources

A variety of C_3 plants were screened for glycolate oxidase (GO) activity. As a result, the GO extracted from *M. falcata* Linn. was found as the best catalyst for oxidation of glycolic acid. Fig. 1 shows the total yield and specific activity of glycolate oxidase from different plant sources. Among those plant sources, *M. falcata* Linn. leaves produced the highest specific activity and optimum yield of GO. Its specific activity was nearly 3.3-fold higher than that of spinach GO. *M. falcata* Linn., which belongs to genus *Medicago* of family *Leguminosae*, is widely distributed in the middle Asia, the northeast, northwest and north of China, and is easily available during major period of every year.

3.2. Properties of amine-functionalized magnetic nanoparticles

Fig. 2 shows the Fourier transform infrared (FT-IR) spectroscopy of the amine-functioned magnetic nanoparticles. The peak at 3423 cm^{-1} is attributed to the –OH group vibration and the peak at 574 cm^{-1} to the Fe–O bond vibration of the Fe_3O_4; the two peaks around 2935 cm^{-1} and 1573 cm^{-1} can be attributed to the stretching vibration of $–CH_2$ and $–NH_2$ groups respectively, which belong to the structure of 1,6-hexanediamine. These characteristic stretching vibrations indicate that the Fe_3O_4 molecules were successfully functionalized with 1,6-hexanediamine and free $–NH_2$ groups were formed on the surface of the magnetic nanoparticles.

The size and morphology of the amine-functioned magnetic nanoparticles were observed by scanning electron microscopy (SEM). Fig. 3(a) illustrates the SEM image of the amine-functioned magnetic nanoparticles which were prepared by hydrothermal synthesis method. It is clearly seen that the size distribution of the magnetic nanospheres was very narrow, with an average diameter of approximately 50 nm.

3.3. Immobilization of GO onto magnetic nanoparticles

The free *M. falcata* GO (MFGO) was used as biocatalyst for the oxidation of glycolic acid to glyoxylic acid and the selectivity of glyoxylic acid production could be as high as 97.6%. However, there were several problems to be addressed for practical application of this enzyme. For instance, the free MFGO was comparatively unsta-

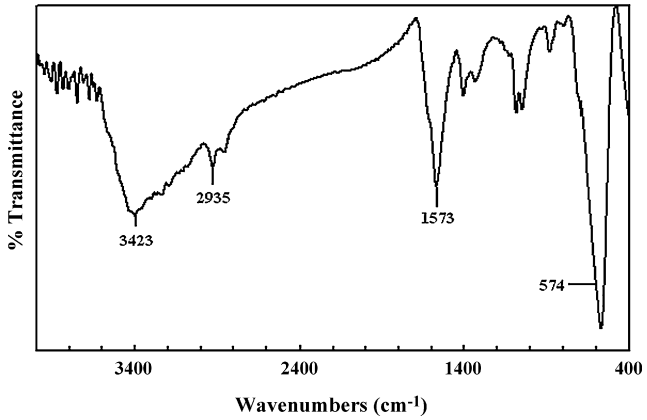

Fig. 2. FT-IR spectrum of the amine-functioned magnetic nanoparticles.

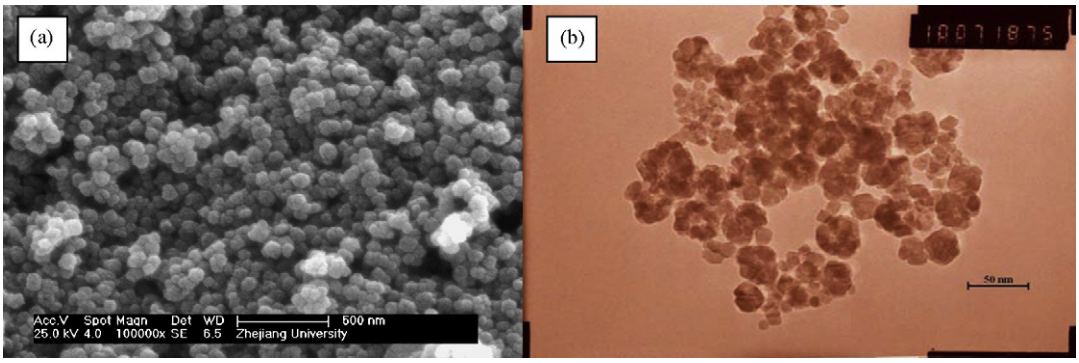

Fig. 3. (a) SEM image of amine-functioned magnetic nanoparticles (~50 nm) and (b) TEM image of the immobilized enzyme.

ble under the reaction conditions and the soluble enzyme was not easy to be used repeatedly. Therefore, we tried to immobilize the MFGO onto amine-functioned magnetic nanoparticles via simple physical adsorption under a mild condition. In order to maximize the MFGO load onto the nanoparticles, effect of the free enzyme dosage on the activity recovery of immobilized MFGO was tested. Fig. 4 shows the adsorption isotherm of glycolate oxidase onto magnetic nanoparticles. The activity recovery reached the maximum (45.2%) when the free enzyme concentration was 10 mg/ml, however the adsorbed protein was only 34 mg/g beads which did not reach the equilibrium until the free enzyme concentration was higher than 30 mg/ml. As presented in Fig. 4, the activity recovery decreased when the enzyme concentration was higher than 10 mg/ml, and it became rather low when the MFGO concentration was 30 mg/ml, which led to a lower total activity of immobilized MFGO. It may be explained by the resistance of mass transfer caused by the immobilization of too much enzyme [32] and the hollow sphere structure of the beads [33]. Thus, the optimum enzyme concentration was chosen as 20 mg/ml, at which the activity recovery reached 36.1% and the adsorbed protein was 70 mg/g beads. The coimmobilization of catalase was also tested, however, the activity recovery of catalase was rather low (<7%). Perhaps the carrier or the immobilization method may not suit for the immobilization of catalase.

The morphology of the immobilized MFGO was observed by transmission electron microscopy (TEM), and the result was presented in Fig. 3(b). The TEM image revealed that this kind of material was a hollow sphere which was worthy to note, owing to its advantage in strong magnetism and large specific area [29].

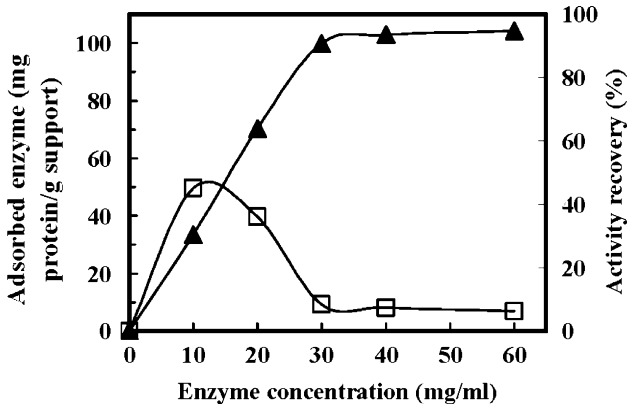

Fig. 4. Effect of the free enzyme dosage on the immobilization of MFGO onto magnetic nanoparticles: protein adsorbed (▲); activity recovery (□). Immobilization condition: 0.5 g amine-functioned magnetic nanoparticles were resuspended in 25 ml Tris–HCl buffer (100 mM, pH 9.0, containing 0.01 mM FMN) by ultrasonication, then added with MFGO and shaken at 15 °C and 160 rpm for 24 h.

3.4. pH and temperature effects on the free and immobilized MFGOs

The effects of pH on the initial activity and stability of free and immobilized MFGOs were tested at the pH ranging from 6.0 to 11.0. The optimum pH of the free and immobilized MFGOs were both 9.0 at 30 °C (data not shown), and the relative activity of the immobilized MFGO did not change so significantly with the pH variation as

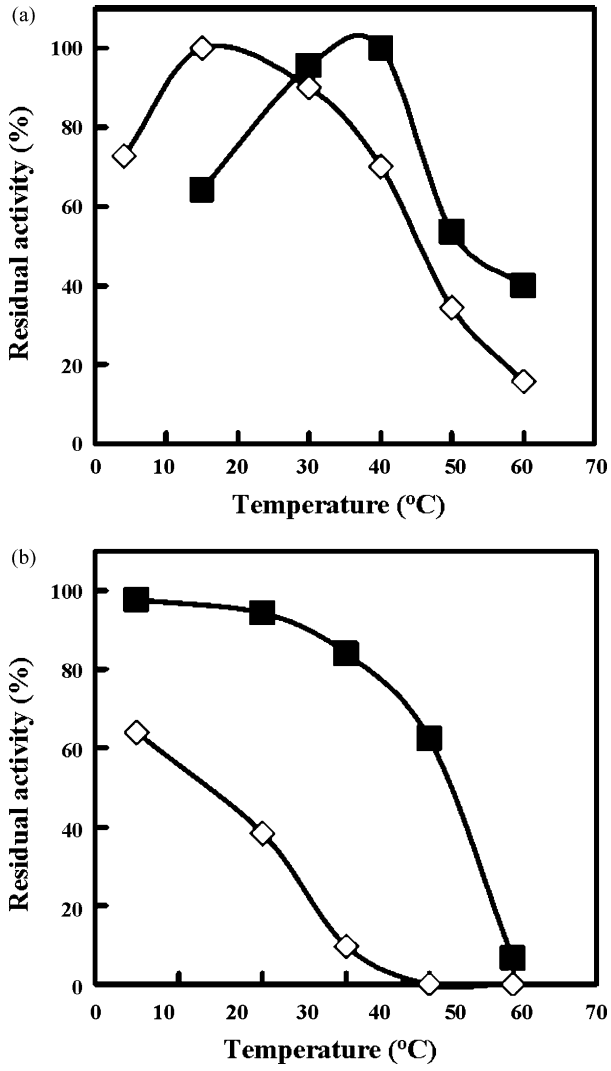

Fig. 5. Effect of temperature on the initial activity (a) and stability (b) of free and immobilized GOs: free GO (◊); immobilized GO (■).

Table 1
Kinetic parameters of free and immobilized GOs.

Form of enzyme	K_m (mM)	V_{max} (mmol/(g protein min))	EL[a] (mg/g support)	Catalytic efficiency (V_{max}/K_m)	Efficiency factor[b]
Free	0.14	0.173	–	1.24	–
Immobilized	5.87	0.122	56.9	0.021	0.71

[a] Enzyme loading (mg protein/g support).
[b] Efficiency factor was defined as the ratio of V_{max} of immobilized GO to free GO.

the free one. The free and immobilized MFGOs were relatively stable at pH 9.0 after incubated at 4 °C for 24 h, retaining about 71.5% and 96.4% of their initial activities respectively.

The effect of temperature on the activity of free and immobilized MFGOs was investigated at a variety of temperatures, from 15 °C to 60 °C. As shown in Fig. 5(a), the free MFGO reached its activity maximum at 15 °C, whereas it was shifted to 40 °C for the immobilized MFGO, which could be attributed to the enhancement of thermal stability resulted by the immobilization. Fig. 5(b) illustrates a comparison of thermal stabilities between the free and immobilized MFGOs. The immobilized MFGO was much less sensitive to the pH and temperature variations. After incubation for 10 h at various temperatures (15–60 °C), the immobilized MFGO retained more activity than the free one. At 50 °C, the free MFGO was inactivated completely after 10 h incubation, while the immobilized MFGO still preserved 62.4% of its initial activity. Since the relative activity of immobilized MFGO at 30 °C was only 5% lower than that at 40 °C while the residual activity after incubation at 30 °C for 10 h was 10% higher than that at 40 °C, we finally chose 30 °C as the optimum reaction temperature for the immobilized MFGO.

3.5. Kinetic parameters of free and immobilized MFGOs

Kinetic parameters of free and immobilized MFGOs were determined by using glycolic acid as substrate at different concentrations. The Michaelis constant (K_m), the maximal initial rate of the reaction (V_{max}) and other related kinetic parameters are presented in Table 1. The K_m value of the free MFGO was 0.14 mM, much smaller than that of the GO from spinach (0.38 mM) [34], implying higher enzyme–substrate affinity of the MFGO. The K_m value of the immobilized MFGO was calculated to be 5.87 mM, almost 42-fold higher than that of the free GO. The V_{max} value of the free MFGO was calculated as 0.173 mmol g protein^{-1} min^{-1}, about 1.4-fold higher than that of immobilized MFGO. These results were considered to be reasonable, since it is well known that immobilization of an enzyme may cause the variation of enzyme kinetic parameters, and these variations can be attributed to several factors resulted from immobilization, such as the steric hindrance, distributional and diffusional effects.

3.6. Reusability of immobilized MFGO for glyoxylic acid production

The operational stability of immobilized MFGO was investigated by the repeated use of immobilized MFGO in a batchwise reactor for oxidization of glycolic acid to glyoxylic acid at 30 °C. EDA and catalase were added in the reaction mixture as reported [9] to improve the yield and selectivity of glyoxylic acid. FMN was also included in the reaction mixture at a concentration of 0.01 mM to enhance the stability of immobilized GO [9].

The batchwise reactions were performed at 30 °C. After each batch of reaction, the immobilized MFGO was isolated by a magnet, washed and added into fresh reaction mixture. The result was shown in Fig. 6. Compared with the reaction time of about 12 h taken in the first cycle, it took more than 17 h for the immobilized MFGO to complete the fourth cycle. The bioactive nanoparticles gradually lose its enzymatic activity as a function of time and the reaction rate

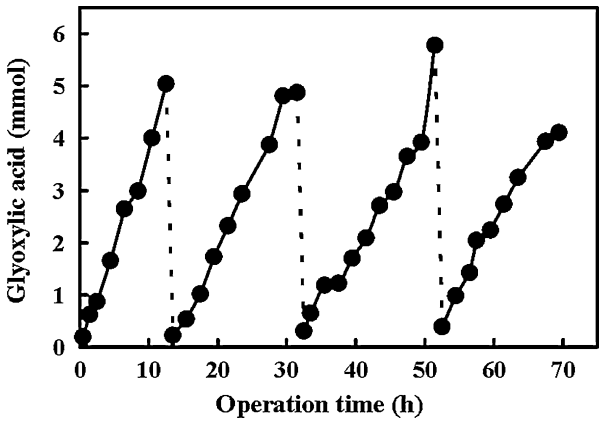

Fig. 6. The batch reaction process of immobilized GO in reactor. Reaction conditions: the mixture of 100 mM glycolic acid, 0.01 mM FMN, 102 mM EDA and 2.0×10^5 IU catalase was stirred with 500 mg immobilized GO in 50 ml Tris–HCl (100 mM, pH 9.0) at 30 °C in a 250-ml batch reactor. Immobilized enzyme was separated from reaction medium by a magnet after each batch of reaction and the washed enzyme was added into a fresh reaction mixture for the next cycle.

became slower. When the reactions were over, 19.8 mmol glyoxylic acid was obtained with slight amount of by-products (formic acid and oxalic acid). The selectivity of glyoxylic acid production was calculated to be as high as 98.9%. The immobilized GO retained 70% of its initial activity after 4 cycles of reaction (nearly 70 h) and the half-life was calculated to be about 117 h.

4. Conclusions

A glycolate oxidase was discovered and isolated from *M. falcata* Linn. which had never been reported before. It showed higher specific activity than other C_3 plants, especially spinach, the well-known GO source. The immobilization of MFGO was carried out subsequently in order to enhance its operational stability and to achieve the reusability in practical application. The amine-functioned magnetic nanoparticles were prepared by hydrothermal synthesis and used for the immobilization of MFGO. Instead of the covalent attachment which was usually used in the immobilization of proteins onto magnetic nanoparticles, we first presented a facile way to prepare the glycolate oxidase–magnetic nanoparticles via mild physical adsorption. The GO retained much more of its initial activity after immobilization onto the amine-functioned magnetic nanoparticles than the GO immobilized on other supports. The advantages in separation, high enzyme loading capacity (56 mg protein/g support) and the excellent operational stability implied the great potential of immobilized GO in large-scale industrial use.

Acknowledgments

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