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Analytical Methods

An innovative method for immobilizing sucrose isomerase on ε-poly-L-lysine modified mesoporous TiO_2

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ABSTRACT

Sucrose isomerase (SIase) is the key enzyme in the enzymatic synthesis of isomaltulose. Mesoporous titanium dioxide ($M\text{-}TiO_2$) and ε-poly-L-lysine-functionalized $M\text{-}TiO_2$ ($EPL\text{-}M\text{-}TiO_2$) were prepared as carriers for immobilizing SIase. SIase was effectively immobilized on $EPL\text{-}M\text{-}TiO_2$ ($SI\text{-}EPL\text{-}M\text{-}TiO_2$) with an enzyme activity of 39.41 U/g, and the enzymatic activity recovery rate up to 93.26%. The optimal pH and temperature of immobilized SIase were 6.0 and 30 °C, respectively. $SI\text{-}EPL\text{-}M\text{-}TiO_2$ was more stable in pH and thermal tests than SIase immobilized on $M\text{-}TiO_2$ and free SIase. K_m of $SI\text{-}EPL\text{-}M\text{-}TiO_2$ was 204.92 mmol/L, and v_{max} was 45.7 μmol/L/s. Batch catalysis reaction of sucrose by $SI\text{-}EPL\text{-}M\text{-}TiO_2$ was performed under the optimal conditions. The half-life period of $SI\text{-}EPL\text{-}M\text{-}TiO_2$ under continuous reaction was 114 h, and the conversion rate of sucrose after 16 batches consistently remained at around 95%, which indicates that $SI\text{-}EPL\text{-}M\text{-}TiO_2$ has good operational stability. Thus, $SI\text{-}EPL\text{-}M\text{-}TiO_2$ can be used as a biocatalyst in food industries.

1. Introduction

Isomaltulose (α-D-glucosylpyranosyl-1,6-D-fructofuranose), commonly referred to as palatinose, is a potential disaccharide sugar substitute and structural isomer of sucrose. Isomaltulose is found in small quantities in honey and sugar cane. Isomaltulose and its hydrogenated derivative, isomaltitol, have attracted worldwide attention because of their function as a food ingredient (Li et al., 2013; Ravaud, Watzlawick, Haser, Mattes, & Aghajari, 2006). Isomaltulose is slowly hydrolyzed by isomaltase and absorbed as glucose and fructose in organism (Haberer, Thibault, Langhans, & Geary, 2009). Contrary to sucrose, isomaltulose can also prevent tooth decay, attenuate insulin levels and the glycemic index in the bloodstream, which indicates its potential as a parenteral nutrient acceptable to diabetics (Hamada, 2002; Kawai, Okuda, & Yamashita, 1985).

Recently, the enzymatic conversion of sucrose to isomaltulose has drawn wide attention using sucrose isomerase (SIase, EC 5.4.99.11), found in many strains. The SIase with molecular weight of 66 kDa hydrolyzes the α-1,2 bond between glucose and fructose and then forms an α-1,6 bond to produce isomaltulose or an α-1,1 bond to produce trehalulose (1-O-α-D-glucopyranosyl-D-fructose) (Cheetham, 1984; Huang, Hsu, & Su, 1998; Véronèse & Perlot, 1999). Several bacterial strains have recently been investigated for isomaltulose production by cell immobilization. The immobilized recombinant *Escherichia coli* cells producing SIase exhibited good stability for 40 days with 83% ± 2% isomaltulose yield (Li et al., 2013). Krastanov, Blazheva, and Stanchev (2007) and Krastanov and Yoshida (2003) reported a 90% sucrose conversion with immobilized *Serratia plymuthica* cells. De Oliva-Neto and Menao (2009) reported that the specific activity of the immobilized *Protaminobacter rubrum* cells ranged from 1.6 g to 4.0 g isomaltulose g/pellet/h with 70% and 65% (w/v) sucrose solution, respectively. Li, Zhao, An, and Zhang (2003) observed that the immobilized *Klebsiella* sp. LX3 cells could convert 99.7% of 10% (w/v) sucrose solution into 87% isomaltulose, but the conversion

Abbreviations: SIase, sucrose isomerase; EPL, ε-poly-L-lysine; $M\text{-}TiO_2$, mesoporous titanium dioxide; $EPL\text{-}M\text{-}TiO_2$, ε-poly-L-lysine-functionalized mesoporous titanium dioxide; $SI\text{-}M\text{-}TiO_2$, sucrose isomerase immobilized on mesoporous titanium dioxide; $SI\text{-}EPL\text{-}M\text{-}TiO_2$, sucrose isomerase immobilized on ε-poly-L-lysine-functionalized mesoporous titanium dioxide.

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rate of sucrose decreased to 71.8% when the sucrose concentration was increased up to 30%. The above immobilized cells, however, also exhibit some deficiencies. For example, space steric hindrance increased compared with free enzyme or cells, and the unit enzyme activity could catalyze fewer substrates. To a certain extent, enzyme immobilization techniques can solve the above problems, but few studies have reported on immobilization of the SIase for the conversion of sucrose to isomaltulose.

Enzyme immobilization techniques can be employed to separate enzymes from the reaction medium easily by simple sedimentation and facilitate the continuous reaction (Fernandez-Lafuente, 2009). More importantly, it also has the potential to improve stabilities and reuse enzymes in numerous bioreactor applications (Cisani, Varaldo, Ingianni, Pompei, & Satta, 1984; Kim, Grate, & Wang, 2006; Moreno & Sinisterra, 1994).

In recent years, many mesoporous materials are popularly used as carriers for enzyme immobilization. Of them, mesoporous titanium dioxide (M-TiO_2), one type of mesoporous materials, is an environmentally friendly and highly stable nanocrystalline material. This material has high surface area-to-volume ratio, good biocompatibility and coordination ability with amine and carboxyl groups (Lori & Nok, 2004; Sadjadi, Farhadyar, & Zare, 2009; Tang, Yan, Tai, & Chan, 2010; Wei, Lu, Ouyang, Yong, & Yu, 2013). These unique properties of M-TiO_2 make it an ideal carrier for enzyme immobilization or attachment of enzymes in enzymatic bioreactors. The enzymes can be combined onto the inner and an outer surface of M-TiO_2, and the amount of enzymes attached to outer surface of carrier relative to that attached to the inside surface of the carrier is negligible (Kotha, Raman, Ponrathnam, Kumar, & Shewale, 1998).

Enzymes immobilized on supports have been considered to occur through electrostatic binding, simple adsorption of enzymes onto their surface, and covalent attachment or encapsulation, which has achieved relatively satisfactory results. Wei et al. (2013) reported the immobilization of β-glucosidase on mercaptopropyl functionalized M-TiO_2. After 10 batches hydrolysis, the cellobiose conversion rates were still around 90%. Wang et al. (2014) chemically modified M-TiO_2 with 3-aminopropyltriethoxysilane, and the immobilization γ-glutamyltranspeptidase exhibited good operational stability. In addition to the above enzymes, as well as alkaline protease and bone morphogenetic protein were immobilized on TiO_2 successfully (Han, Jang, Kim, & Koh, 2014; Sadjadi et al., 2009).

In this paper we investigated the immobilization of SIase on M-TiO_2 and ε-poly-L-lysine-mesoporous titanium oxide (EPL-M-TiO_2), the biochemical characteristics of free and immobilized enzyme and the conversion of sucrose to isomaltulose and trehalulose. Therefore, a rational strategy of a more specific and efficient immobilized enzyme for economical production of isomaltulose in food industry is provided. Previous studies (Huang et al., 1998; Lee, Kim, Kim, & Lee, 2008; Li et al., 2013) had shown that isomaltulose was always produced with SIase in the form of free enzyme or immobilized cells. This study successfully immobilized the SIase on the surface of EPL-M-TiO_2, and it is expected to make its contribution to the immobilization technology.

2. Materials and methods

2.1. Materials

EPL was produced by *Streptomyces albulus* PD-1 strain, with a molecular weight of approximately 2.5–3.5 kDa (Xia, Xu, Feng, Xu, & Chi, 2013). M-TiO_2 was provided by Lu (He, Lu, Feng, Yu, & Yang, 2004). The other reagents and chemicals used in this study were purchased from Aladdin Industry Co., Ltd. (Shanghai, China).

2.2. Production and purification of SIase

SIase from *E. coli* (pET22b-*pal*I) using fermentation medium, induced with 0.5 mM lactose (Li et al., 2011). The cells were decomposed by ultrasonic, carried out in buffer A (10 mM Hepes, 500 mM NaCl, and 10% glycerol, pH 8.0), after washed with buffer B (50 mM, pH 6.0 phosphate buffer). Then cell disruption liquid was centrifuged to remove the debris of cells. A Ni–NTA resin column was used to load the filtrate, which was equilibrated with buffer A. Then the column was washed by the buffer A containing 50 mM imidazole, and a gradient of imidazole (from 100 mM to 300 mM) was applied to elute enzymes. The enzyme solution was dialyzed overnight against buffer B with 10% glycerol, and the dialysate was stored at 4 °C. The purity of SIase was determined by SDS–PAGE analysis.

2.3. Preparation of enzyme carriers M-TiO_2 and ELP-M-TiO_2

The M-TiO_2 powder was mixed with buffer B and ultrasonically dispersed for 30 min. Then, the M-TiO_2 powder was separated using vacuum suction filtration. The M-TiO_2 powder (1 g) was dispersed in 40 mL buffer B with 3.0% (w/w) EPL. The mixture was incubated at 25 °C for 10 h. After filtration, the powders (EPL-M-TiO_2) were washed thrice with buffer B.

2.4. Immobilization of SIase on M-TiO_2 and EPL-M-TiO_2

1 g M-TiO_2 (or EPL-M-TiO_2) powders were put in different concentrations of 40 mL SIase solution (0.15–1.35 U/mL), respectively. The mixture was then stirred at 25 °C for 6 h. Then, the enzyme to be immobilized on M-TiO_2 or EPL-M-TiO_2 (SI-M-TiO_2 or SI-EPL-M-TiO_2) was separated using vacuum suction filtration, and the filter liquor was preserved for the determination of enzyme activity. To remove the untrapped enzyme molecules, the immobilized enzymes were washed with buffer B until no SIase activity could be detected in washings. The washings were collected to estimate the amount of enzyme bound on the carriers. The percentage of immobilized SIase activity to initial SIase activity in solution is defined as the enzymatic activity recovery.

2.5. Structural characteristics of M-TiO_2 and EPL-M-TiO_2

Scanning electron microscopy (SEM, S-4800, Hitachi, Japan) was used to investigate the surface morphology of M-TiO_2 and EPL-M-TiO_2. Fourier-transform infrared spectroscopy (FTIR, PerkinElmer Spectrum 100 instrument) was conducted to determine the combination of EPL and M-TiO_2.

2.6. Enzyme assay

2.6.1. Free enzyme

The reaction mixture was composed of 0.8 mL 50 g/L (w/v) sucrose in buffer B and 0.2 mL of appropriately diluted SIase. The reaction was incubated at 30 °C for 20 min, which was stopped by boiling for 10 min, and centrifuged at 12,000×*g* for 5 min to remove denatured proteins. The amount of isomaltulose in the supernatant was determined by high-performance liquid chromatography (HPLC, Agilent 1200, USA system equipped with a refractive index detector). One unit (U) of SIase activity is defined as the amount of enzyme that forms 1 μmol of isomaltulose per minute under standard assay conditions.

2.6.2. Immobilized enzyme

Immobilized enzyme activity was measured by incubating 0.025 g of immobilized enzyme with 1 mL of 40 g/L (w/v) sucrose in buffer B at 30 °C for 20 min and stopped by centrifuging at

12,000×g for 1 min. Thus, 1 U of immobilized enzyme activity is defined as the amount of SIase that releases 1 μmol isomaltulose per minute under standard assay conditions.

2.7. Sugar composition

Prior to HPLC analysis, the reaction mixture was filtered by 0.22 μm membrane filters. The samples were appropriately diluted, and 20 μL diluted sample was injected onto a BP-100 Pb^{2+} column (10 μm, 300 mm × 7.8 mm) to measure sugar composition. The mobile phase was HPLC grade water with a flow rate of 0.4 mL/min at 80 °C. Sugar components were analyzed with a refractive index detector (RI-101). The HPLC and BP-100 Pb^{2+} column were purchased from Agilent (Wilmington, DE) and Benson (Beijing, China).

2.8. Properties of immobilized and free enzymes

The optimal temperature of free and immobilized enzymes was investigated from 10 °C to 60 °C at pH 6.0. The thermal stability of SIase was evaluated by incubating the enzyme at different temperatures (10–60 °C) at pH 6.0 for 4 h. To determine the optimal pH, enzyme assay was investigated from pH 4.5 to pH 8.0 in citric acid-phosphate buffer at 30 °C. Similarly, the pH stability of SIase was examined by incubating the enzyme at different pH levels (4.5–8.0) at 4 °C for 36 h. Unless specified, all reactions were performed as the method of enzyme assay. The residual activity of free and immobilized enzyme was estimated under standard assay conditions.

2.9. Determination of kinetic parameters

The kinetic parameters of immobilized and free enzyme were determined by measuring the rate of reaction with an increasing concentration of sucrose (35–840 mM). The reactions were conducted in buffer B, at 30 °C for 20 min. The Michaelis–Menten Constant (K_m) and maximum reaction velocity (v_{max}) values were calculated using the Lineweaver–Burk equation.

2.10. Reusability of the immobilized enzymes

The batch reaction was performed with 20 mL sucrose solution (50 g/L) and 0.5 g SI-M-TiO_2 (or SI-EPL-M-TiO_2) in a 50 mL Erlenmeyer flask. The reactions were incubated in a shaking bath at 30 °C until the substrate was completely catalyzed to products. SI-M-TiO_2 and SI-EPL-M-TiO_2 were used repeatedly, which were washed with buffer B after every batch. All samples of every batch were analyzed by HPLC after incubation.

2.11. Statistical analysis

Data were expressed as the means ± standard deviation. Statistical analysis was conducted by one-way ANOVA followed by Duncan's test ($P < 0.05$) using the SPSS 17.0 software.

3. Results and discussion

3.1. Purification of the recombinant SIase

SDS–PAGE analysis of purified proteins revealed a single polypeptide with the presence of abundant protein around 66 kDa (Data not shown). After enrichment, the activity of purified enzyme was 128.4 U/mL under optimal conditions.

3.2. M-TiO_2 modified with EPL

Surface of M-TiO_2 is lined with dense hydroxyl groups, which provides the support of M-TiO_2 to carry negative charges, under physiological pH conditions (Wang et al., 2014). Due to the presence of abundant free amino groups, EPL carries positive charges (Xia et al., 2013), whereas SIase also carries negative charges (Ravaud et al., 2007). Therefore, the modified M-TiO_2 was embedded by EPL, changed the characteristics of the surface of the M-TiO_2 with negative charges. The above indicated that EPL, one of the polycation biological materials, was used to modify M-TiO_2 in physiological pH solutions, which could not only improve the biocompatibility between enzyme and M-TiO_2, but also increase the amount of enzyme loading on the surface of M-TiO_2 due to the electrostatic adsorption between positive and negative charges. Thus, it could reduce the resistance of combination between enzyme and substrate. All the above characteristics were attributed to microenvironmental effect, under the existence of EPL, the microenvironment of enzyme changed (Venkataraman, Horbett, & Hoffman, 1977).

3.3. Structural characteristics of M-TiO_2 and EPL-M-TiO_2

M-TiO_2 was characterized by SEM and FTIR before and after being functionalized, respectively. In Fig. 1, SEM results of the surface morphologies analysis of M-TiO_2 and EPL-M-TiO_2 were shown. By contrast, after modification, the shape of the surface has changed significantly, and EPLs in the form of social covering were observed on the surface of M-TiO_2.

FTIR spectra were recorded for native and EPL treated M-TiO_2 to understand the interaction between EPL and M-TiO_2 (Fig. 2). The FTIR spectra shows the characteristic absorption peak of Ti–O–Ti at approximately 495 cm^{-1}, and the stretching vibration peak near 3419 cm^{-1} is due to the hydroxyl group of M-TiO_2 (Wang et al., 2014). The C═O stretch vibration of amide bond is near 1668 cm^{-1}. The peak at 1537 cm^{-1} corresponds to C—N—H stretching vibration of amido bond, whereas the peak at 1100 cm^{-1} shows the C—N stretching of the amido bond (Liang, Yuan, Liu, Wang, & Gao, 2014; Shima & Sakai, 1977). The peak at approximately 3150 cm^{-1} shows the N—H stretching that corresponds to the free amino group (Rao et al., 2012). The SEM and FTIR results show the existence of amine group and amido bond at the carrier surface and also indicate that EPL has been combined with M-TiO_2, successfully.

3.4. Immobilization of SIase on M-TiO_2 and EPL-M-TiO_2

SIase was immobilized on M-TiO_2 and EPL-M-TiO_2 at the initial concentration of 0.15 U/mL to 1.35 U/mL free SIase in solution. With the increase in the initial enzyme concentration, the activity of SI-M-TiO_2 gradually increased (Table 1). However, the enzyme activity recovery rate declined precipitously. When the initial enzyme concentration was greater than 0.75 U/mL, the activity of SI-M-TiO_2 did not show obvious difference with the increase in the initial enzyme concentration, and SI-M-TiO_2 activity was basically stable at about 8.2 U/g. Inversely, increasing the initial enzyme concentration resulted in a rapid increase in the activity of SI-EPL-M-TiO_2. When the initial SIase concentration was 1.05 U/mL, SI-EPL-M-TiO_2 activity reached up to 39.41 U/g. However, further increase in the initial concentration of enzyme sharply reduced SI-EPL-M-TiO_2 activity. This phenomenon is caused by the increase in the amount of enzyme loading on the carrier, which causes the space steric effect. Caldwell, Axen, Bergwall, Olsson, and Porath (1976) reported that the activity of immobilized enzyme was determined by the load of enzymes on the carriers without space steric hindrance. Additionally, the

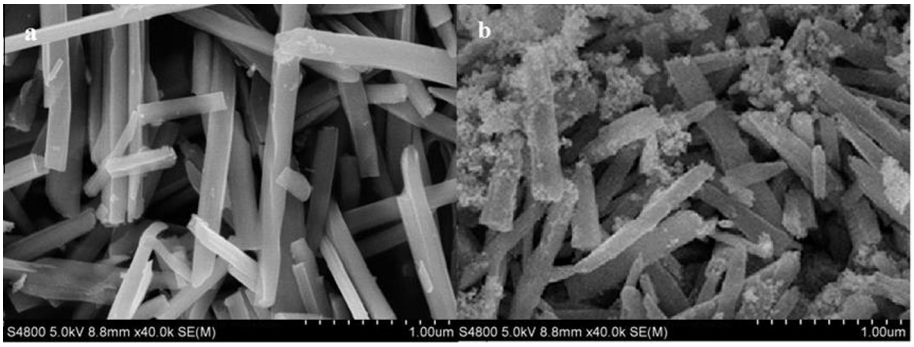

Fig. 1. SEM images of (a) M-TiO_2 and (b) EPL-M-TiO_2.

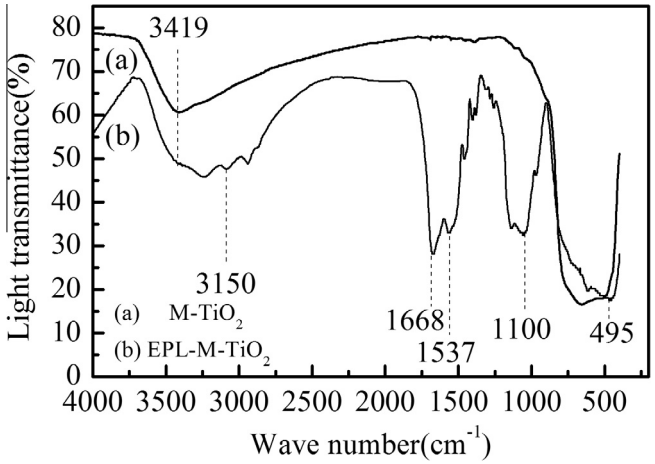

Fig. 2. FTIR spectra of (a) M-TiO_2 and (b) EPL-M-TiO_2.

electrostatic interaction among the enzymes probably changed the conformation of enzyme. EPL-M-TiO_2, the EPL functionalized carrier, showed higher enzymatic activity recovery than the native M-TiO_2. The highest enzymatic activity recovery of SI-M-TiO_2 was 83.75% at initial SIase concentration of 0.15 U/mL. The highest enzymatic activity recovery of SI-EPL-M-TiO_2 was 93.26% at initial SIase concentration of 1.05 U/mL. Selecting the highest enzymatic activity recovery of immobilized operation, the activity of SI-EPL-M-TiO_2 (39.41 U/g) was approximately eightfold as much as SI-M-TiO_2 (5.04 U/g). It was obvious that the amount of enzyme loadings on M-TiO_2 and EPL-M-TiO_2 was very remarkable. EPL is conducive to the immobilization of SIase for its good heat stability, water solubility and unique structure linking α-carboxylic and ε-amino groups, especially the free amino groups it contains (Xia et al., 2013; Zhang et al., 2012). Thus, the EPL can simultaneously improve the biocompatibility between carriers and enzymes and hence enhance their adhesion strength. So the above indicated that the EPL changed the nature of the charges at the surface of M-TiO_2, which could avoid the repulsion between the same charges and prompt the electrostatic adsorption between different charges. Furthermore, EPL can reduce the risk of microbial contamination of sugar solution during isomaltulose production from sucrose (Bo et al., 2014).

3.5. The properties of immobilized and free SIase

3.5.1. Optimal pH and temperature

The optimization of pH is a significant step for increasing the activity of both immobilized and free enzymes. The pH-dependent activities of immobilized and free enzyme were determined at pH 4.5 to 8.0 (Fig. 3a). As is shown no significant changes are observed at the optimal pH (6.0) of free and immobilized SIase. However, SIase immobilized by EPL-M-TiO_2 had a wider pH range. The effect of temperature on enzyme activity of immobilized and free enzymes were performed from 10 °C to 60 °C (Fig. 3c). The optimal temperature for the reaction of three enzymes was 30 °C. Compared with that of the other two enzymes, the activity of SI-EPL-M-TiO_2 was more active at high temperature with noticeable broadening of the optimal range. From 15 to 45 °C, the relative activity of SI-EPL-M-TiO_2 was more than 90%. For isomaltulose production from sucrose, isomerization reaction conducted from 30 °C to 40 °C is the most suitable temperature for industrial isomaltulose production, because the physiological temperature do not cause the inactivation of enzyme and can save energy. Compared with other catalytic reaction temperature, 40 °C is a relatively high temperature, which is beneficial to the production of isomaltulose (Li & Xu, 2011). When the temperature was 60 °C, the relative activity still maintained more than 70%. However, the activity of free enzyme was almost denatured at 60 °C. These results show that the range of optimal pH and temperature has been improved, compared with free SIase and SI-M-TiO_2 (Fig. 3c). Furthermore, pH 6.0 as the optimal pH of immobilized SIase meets the requirement of industry to maintain the pH value within 6.0–6.5, which can remarkably control the browning changes (Rhimi et al., 2009).

Table 1
Effect of the initial enzyme concentration on immobilized SIase.

Initial SI activity in the free SI solution (U/mL)	Immobilized enzyme activity (U/g)		Activity in the supernatant (%)		Actual enzymatic activity recovery rate (%)	
	SI-M-TiO_2	SI-EPL-M-TiO_2	SI-M-TiO_2	SI-EPL-M-TiO_2	SI-M-TiO_2	SI-EPL-M-TiO_2
0.15	5.04 ± 0.12a	3.75 ± 0.01a	14.14 ± 0.09a	35.68 ± 1.9c	83.75 ± 0.51e	61.28 ± 0.21a
0.45	7.21 ± 0.52b	11.71 ± 0.12b	59.02 ± 0.41b	33.44 ± 2.4bc	39.44 ± 0.18d	64.70 ± 0.35b
0.75	8.21 ± 0.77bcd	20.48 ± 1.61c	71.09 ± 1.21c	30.70 ± 0.8b	27.04 ± 0.51c	67.94 ± 1.91c
1.05	8.46 ± 0.08c	39.41 ± 0.08e	77.42 ± 0.25d	4.04 ± 0.01a	19.34 ± 0.10b	93.26 ± 0.33d
1.35	8.70 ± 0.05d	35.69 ± 0.52d	80.79 ± 3.63d	31.42 ± 0.11b	15.57 ± 0.06a	65.75 ± 1.00bc

Each value represents the mean ± standard deviation. Different lowercase letters of each column indicate significant difference at $P < 0.05$.

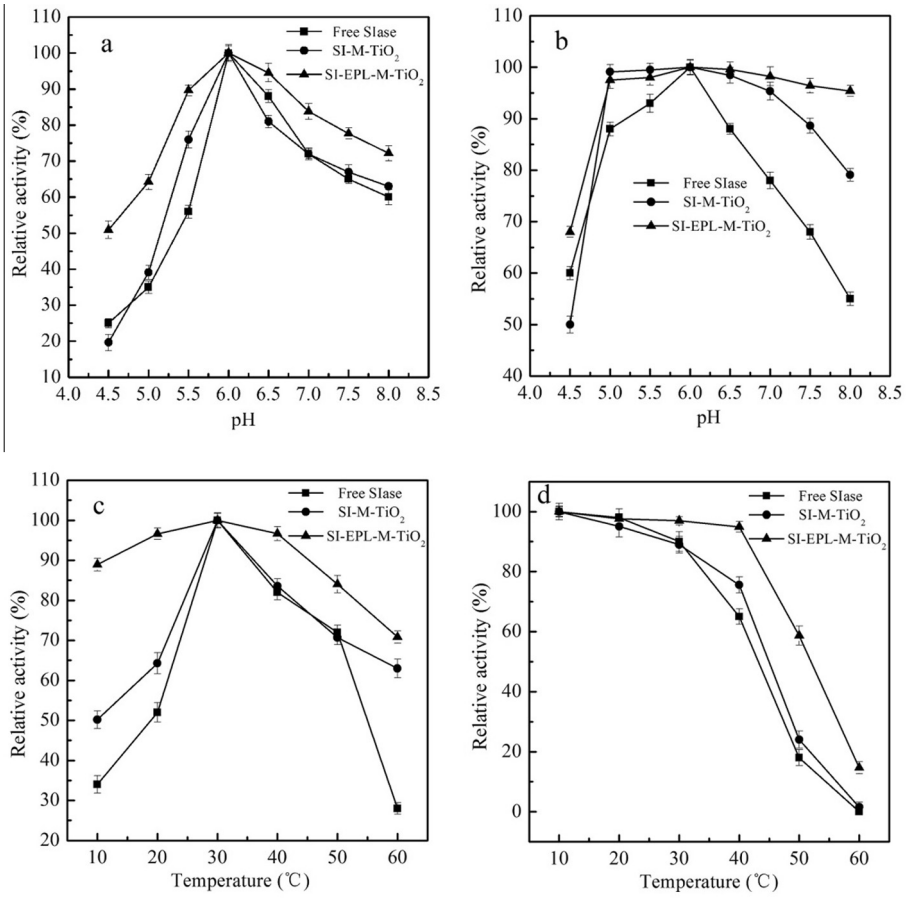

Fig. 3. Effects of (a) pH and (c) temperature on the activity of immobilized and free SIase. (b) pH stability of immobilized and free SIase. (d) Thermal stability of immobilized and free SIase. (■) Free SIase, (●) SI-M-TiO_2 and (▲) SI-EPL-M-TiO_2. The highest activity was defined as 100%.

The wide pH and temperature ranges offer elasticity of operation at the industrial production. And the optional pH and temperature make the SI-EPL-M-TiO_2 a desirable immobilized enzyme for isomaltulose production.

3.5.2. Thermal and pH stability

Thermal stability was tested by measuring the activity after incubation at pH 6.0 from 10 to 60 °C for 4 h. As shown in Fig. 3d, the immobilized (SI-M-TiO_2 and SI-EPL-M-TiO_2) and free enzyme show similar stability at 10–20 °C. However, at incubation temperatures over 20 °C, the thermal stability of SI-EPL-M-TiO_2 was better than that of free SIase and SI-M-TiO_2. Moreover, the residual activity of SI-EPL-M-TiO_2 still remained 60% after incubation at 50 °C for 4 h. However, the others were both below 30%. Increasing the reaction temperature can remarkably increase the reaction rate. The carriers are often helpful in improving the stability of the enzyme with a hydrophilic environment (Hwang et al., 2004). EPL contains extensive hydrophilic groups (amino groups), which provides the hydrophilic environment for enzyme immobilization. EPL-M-TiO_2 provides a high amino density for an efficient immobilization of SIase through many binding sites. SIase is most likely associated with direct interactions between the EPL and the SIase, which can stabilize its native conformation, increase the conformational rigidity of SIase and protect the SIase against denaturation at a higher temperature (Mahmoud, Lam, Hrapovic, & Luong, 2013). The stability of different pH values was determined with free SIase, SI-M-TiO_2 and SI-EPL-M-TiO_2 by measuring the residual activity of SIase after incubation at pH 4.5 to 8.0 for 36 h. As shown in Fig. 3b, SI-M-TiO_2 and SI-EPL-M-TiO_2 were more stable than free SIase, maintaining more than 95% activity at pH 4.8–7.2. However, free SIase retained 95% activity at pH 5.8–6.3, which is a narrow pH range. These results show that the range of thermal and pH stability of SIase immobilized on EPL-M-TiO_2 is expanded to a certain degree (Fig. 3b).

3.6. Kinetic parameters determination

K_m and v_{max} values were determined using Lineweaver–Burk equation by varying the sucrose concentrations at the same temperature and pH. The SI-EPL-M-TiO_2 shows the lowest K_m value in the three enzymes (Table 2). This indicates that SI-EPL-M-TiO_2 has the highest affinity for sucrose, and the v_{max} value matches free SIase. Compared with free SIase, the affinity for sucrose of SI-M-TiO_2 decreases sharply, and the v_{max} is also slightly reduced. The decreased K_m value for SI-EPL-M-TiO_2 indicates that EPL may induce SIase directional immobilization on carriers and EPL may make the enzyme active site contact with the substrate rapidly, as well as the product can quickly leave. Given that SIase entered

Table 2
Apparent kinetic parameters of immobilized and free SIase.

Types of enzyme	K_m (mmol/L)	v_{max} (μmol/L/s)
Free enzyme	259.71 ± 7.78b	46.03 ± 0.87b
SI-M-TiO_2	435.55 ± 20.39c	32.14 ± 1.93a
SI-EPL-M-TiO_2	204.92 ± 4.30a	45.71 ± 1.46b

Each value represents the mean ± standard deviation. Different lowercase letters of each column indicate significant difference at $P < 0.05$.

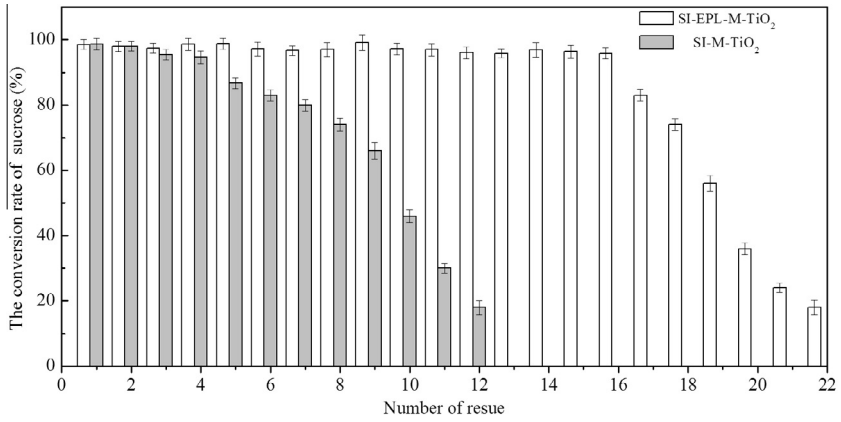

Fig. 4. Number of cycles of the immobilized enzymes that catalyzed sucrose into isomaltulose and trehalulose. (□) SI-EPL-M-TiO_2 and (■) SI-M-TiO_2. The first sucrose conversion rate was defined as 100%.

the channel of M-TiO_2, the increased K_m value and the decreased v_{max} value for SI-M-TiO_2 might have resulted from the steric hindrance and diffusion limitation.

3.7. Repeated enzymatic conversion of sucrose to isomaltulose and trehalulose using immobilized enzymes

Strong reusability of immobilized enzymes is quite important, because it is a crucial quality to be used as industrial catalysts (Goradia, Cooney, Hodnett, & Magner, 2006). A repeated batch catalytic reaction was designed to determine the practical reusability of SI-M-TiO_2 and SI-EPL-M-TiO_2 under optimal conditions (Fig. 4). The two kinds of immobilized SIase could catalyze 50 g/L of sucrose into isomaltulose and trehalulose. The SI-M-TiO_2 shows a general operational stability, the sucrose conversion rate is decreased by less than 90% after 5 batches. The sucrose conversion rate of SI-M-TiO_2 is 65% at the end of the 8th recycle, and the half-life is about 55 h. Inversely, the sucrose conversion rate remains at approximately 95% by SI-EPL-M-TiO_2 after 16 conversion cycles. However, the conversion rate decreases to 50% after 19 cycles, and the half-life is about 114 h. The decreased conversion rate of SI-M-TiO_2 upon reuse is due to the weakening of the interaction force between the carrier and enzyme, which may lead to enzyme fall off from the carrier. Another reason for this phenomenon is the continual encountering of active site and substrate, which may be responsible for the conformational changes and hence the catalytic activity is either partially or completely lost. These results can also confirm that the SIase immobilized by EPL-M-TiO_2 has great operational stability, and EPL plays an irreplaceable role in the process. The operational stability of SI-EPL-M-TiO_2 is stronger than that of the immobilized cells reported previously. The immobilized enzyme SI-EPL-M-TiO_2 containing 1 U of SIase can catalyze the conversion of 0.81 g of sucrose into isomaltulose and trehalulose, which is much better than other immobilization methods. Li et al. (Li et al., 2013) reported that immobilized recombinant *E. coli* cells containing 1 U of SIase could catalyze the conversion of 0.18 g of sucrose. According to the results of the batch catalytic reaction, the high sucrose conversion of one unit of enzyme activity and the strong stability of SI-EPL-M-TiO_2 prove that the proposed immobilization method has potential to be used for the industry to produce palatinose.

4. Conclusions

Enzyme loading capacity is enhanced by functionalizing M-TiO_2 with EPL. The resulting EPL-M-TiO_2 can effectively immobilize SIase, with considerably improved pH, thermal, and operational stability. The novel biocatalyst material, a nanostructured crystalline, can be easily separated from the reaction medium by natural settling or low-speed centrifugation. SI-EPL-M-TiO_2 showed excellent reusability in enzymatic catalysis system over 16 successive cycles. M-TiO_2 functionalized by EPL has great potential to be used in other enzyme systems and can be applied as a practical enzymatic bioreactor in food industries.

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