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Immobilization of phenylalanine dehydrogenase onto Eupergit CM for the synthesis of (S)-2-amino-4-phenylbutyric acid

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ABSTRACT

An oxidoreductase enzyme, phenylalanine dehydrogenase (PheDH) from *Rhodoccocus sp.* 4 was covalently immobilized onto a commercially available enzyme carrier, Eupergit CM and tested for the synthesis of an unnatural amino acid, (S)-2-amino-4-phenylbutyric acid (S-APBA). Through performance evaluation of the immobilization based on its yield and efficiency, an adequate combination of the immobilization conditions to obtain optimum values in these parameters were determined as enzyme loading of 60:1 (weight ratio of enzyme carrier to enzyme) in an immobilization buffer of 500 mM at pH 8.5 for 24 h. Upon covalent immobilization onto Eupergit CM, the operational pH range for reductive amination was slightly broadened (pH 7.5–9.5). Immobilization also helps to improve the thermostability of PheDH, raising the optimum temperature to 50 °C. A remarkable improvement in operability and storage stability was achieved. The immobilized PheDH was successfully applied for the synthesis of (S)-2-amino-4-phenylbutyric acid, achieving enantiomeric excess of more than 99% and yield of more than 80%; comparable to synthesis using the free PheDH. Reductive amination using the immobilized PheDH is beneficial to enhance the asymmetric synthesis S-APBA.

1. Introduction

Enantiopure chiral unnatural amino acids are applauded as indispensable tools in drug discovery, being one of the key components in pharmaceuticals. One of the most significant applications of (S)-2-amino-4-phenylbutyric acid [S-APBA, L-homophenylalanine (L-HPA)] is as a key chiral pharmaceutical drug intermediate. S-APBA acts as a common building block for the synthesis of novel pharmaceuticals such as angiotensin-converting enzyme (ACE) inhibitors and neutral endopeptidace (NEP) inhibitors for the management of hypertension and cardiovascular disease, acetylcholinesterase inhibitors for treatment of Alzheimer and Lewy body dementia, and β-lactam antibiotics for treatment of susceptible bacterial infections. Virtually all ACE inhibitors have the chiral S-APBA moiety presents as the central pharmacophore unit [1].

Numerous scientific explorations have been reported, aiming at developing conceivable processes of synthesizing enantiomerically pure S-APBA. The processes could be classified into two common routes; chemical or biocatalytic. The chemical routes include stereoselective hydrogenation, asymmetric hydrogenation and carbon—carbon bond formation; whereas the biocatalytic routes comprise chiral racemate resolution [2–4] and asymmetric synthesis [5,6]. A comprehensive review on the biocatalytic synthesis of S-APBA [7] revealed that asymmetric synthesis is preferred over racemate resolution for S-APBA synthesis. The asymmetric synthesis could achieve a theoretical yield of 100%, indicating it is a relatively more economical process with lower complexities.

Most of the published biocatalytic routes reported on employment of whole cells or isolated enzymes for synthesis of S-APBA, utilizing different starting materials via reductive amination [8,9], aminotransferase [6], dehydrogenation [10] and hydantoinase/carbamoylase [11,12] processes. Yen et al. [13] reported an improved bi-enzyme process for synthesis of S-APBA using immobilized *N*-acylamino acid racemase and immobilized *N*-carbamoyl-L-amino acid amidohydrolase. Immobilization is typically employed to fully exploit the technical and economical advantages of an enzyme [14]. This plausible modification method of a biocatalyst is deemed effective for performance enhancement and operational simplification for the biocatalyst, besides improving its reusability and stability [15].

The present paper reports the immobilization of phenylalanine dehydrogenase onto Eupergit CM, a copolymer of methacrylate, crosslinked and carrying oxirane groups; targeting to develop an improved biocatalyst for the asymmetric synthesis of S-APBA. Comparisons based on protein immobilization yield and immobilization efficiency (enzyme activity yield) were done for evaluation of the

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immobilization processes, alongside enzyme operational and storage stability.

2. Materials and methods

The enzyme phenylalanine dehydrogenase, PheDH from *Rhodococcus* species strain M4 (E.C. 1.4.1.20) and NAD-specific format dehydrogenase, FDH from *Candida boidinii* (E.C. 1.2.1.1) were purchased from ASA-Spezialenzyme, Germany. NADH was obtained from Roche Diagnostics, Germany.

2-oxo-4-phenylbutanoic acid (OPBA) was purchased from Shanghai Zillionaire Chemicals Inc, China; Eupergit CM was obtained from Sigma–Aldrich. Solvents used as mobile phases in high performance liquid chromatography (HPLC) were of HPLC grade. All other chemicals used were of analytical grade.

2.1. Immobilization of PheDH onto Eupergit CM

The preparation of the immobilized PheDH was carried out on Eupergit CM; a copolymer of methacrylate, crosslinked and carrying oxirane groups. An immobilization protocol modified from a standard method previously reported [16] was adopted in this work, in which the enzyme was directly bound onto support via oxirane groups. 1 mg/mL of soluble PheDH solution was added into a predetermined amount of the polymeric beads. The mixture was mixed thoroughly using a vortex mixer and left for standing overnight without stirring or shaking. Throughout the immobilization period, 5 μL of supernatant was collected at every 12 h interval for the protein concentration analysis and the enzyme activity assay. After the incubation period, the beads were washed 3 times with a cold buffer solution of higher ionic strength [17]. The immobilized enzyme was collected by vacuum filtration using sintered filter glass P1. Samples were drawn from the washing solutions to check for residual protein concentration and enzyme activity.

The immobilization performance was evaluated by immobilization yield and immobilization efficiency, as described in the following sub-sections (Sections 2.2 and 2.3).

Effects of the immobilization conditions that were investigated are as follows:

(i) The ratio of enzyme carrier to enzyme (10–100 mg of support/mg of protein),
(ii) The different buffers and pH (sodium acetate buffer at pH 4, potassium phosphate buffer at pH 7, Tris–HCl buffer at pH 8.5 and Glycine buffer at pH 10),
(iii) The ionic strength of the buffers (10 mM, 100 mM, 500 mM and 1000 mM),
(iv) The incubation time (12–72 h).

2.2. Protein concentration analysis

Bradford method was adopted for the protein concentration determination. Bovine serum albumin (BSA) was used as a protein standard for calibration [18]. This concentration analysis served two purposes; for enzyme specific activity analysis and immobilization yield calculation. Enzyme specific activity is expressed as enzyme activity units per mg of protein, reported for optimal assay conditions at a fixed temperature with all substrates present at saturating concentrations.

Protein concentration in the supernatant and washings were determined for calculation of immobilization yield or enzyme loading on the carriers. The difference between the protein content in the control and the protein concentration in supernatant and washings represents the amount protein bound to carriers. The immobilization yield is calculated according to Eq. (1):

$$\text{Immobilization yield}, \% = 100\% \times \frac{(C_i - C_s)}{C_i} \quad (1)$$

where C_i is the initial protein concentration and C_s is the total protein concentration in the supernatant and all the washing solutions.

2.3. Enzyme activity assay

Enzyme activity assay adopted was modified from a method previously described [19], focusing on the reductive amination reaction only. The assay mixture of 3.0 mL consists of 0.4 mM NADH, 5 mM OPBA, 750 mM NH_4OH–HCl (pH 8.5 at 25 °C) and a limiting amount of enzyme solution. Aliquot of the assay mixture was incubated at 25 °C. Photometrical measurement to follow the decrease of absorbance at 340 nm was monitored for 5 min after adding the enzyme. A blank sample for correcting the readings was prepared with the absence of the enzyme.

The enzyme activity unit is expressed as the consumption of 1 μmol of NADH in a minute, calculated using Eq. (2):

$$\frac{\text{Units}}{\text{mL enzyme}} = \left[\frac{\Delta A_{340\,\text{nm}}/\text{min}\quad \text{sample} - \Delta A_{340\ \text{nm}}/\ \text{min}\quad \text{blank}(V)(\text{df})}{\varepsilon\quad \nu}\right] \quad (2)$$

where $A_{340\,\text{nm}}$ is the change in absorbance at 340 nm, V is total volume of assay (mL), ν is volume of enzyme (mL), df = dilution factor and ε is the milimolar extinction coefficient of β-NADH at 340 nm (6220 $\text{mol}^{-1}\,\text{cm}^{-2}$).

The theoretical activity corresponding to the amount of enzyme bound onto the support is calculated as the difference between the enzyme activity of the initial amount added (A_i) and the enzyme activity in the supernatant and the washings solutions (A_s) [20]. The immobilization efficiency is reported as following:

$$\text{Immobilization efficiency}, \% = 100\% \times \frac{(A_i - A_s)}{A_i} \quad (3)$$

2.4. Characterization of catalytic properties of the enzyme

The characterization study was carried out for soluble and immobilized PheDH. Effects of the pH and the temperature on enzyme stability and activity were investigated.

2.4.1. Effect of the pH on enzyme stability and activity

The effect of the pH on the enzyme activity was evaluated at pH ranging from 4 to 11. The enzymes were pre-incubated in 10 mM buffer for 30 min at 25 °C, before an aliquot of the solution was drawn for enzyme activity determination as detailed out in Section 2.3.

2.4.2. Effect of the temperature on enzyme stability and activity

The thermostability study was carried out by pre-incubating the enzyme at various temperatures (−20, 4, 25, 40, 50, 60 °C) for 30 min prior to conducting the enzyme activity assay according to Section 2.3.

2.4.3. Reusability and storage stability studies

The reusability study of the immobilized PheDH in batch reaction was done by determining the residual activity of the enzyme after each reaction cycle. The enzymes were recovered by filtration after each cycle of 1 h reaction time. The recovered enzymes were rinsed thoroughly with 50 mM pH 8.5 Tris–HCl buffer.

Assay similar to procedure described in Section 2.3 was applied for storage stability studies of PheDH at room temperature (25 °C), 4 °C and −20 °C. The residual activities were measured over a course of 28 days of storage time. Both soluble and immobilized enzymes were stored in 51% wt/wt glycerol.

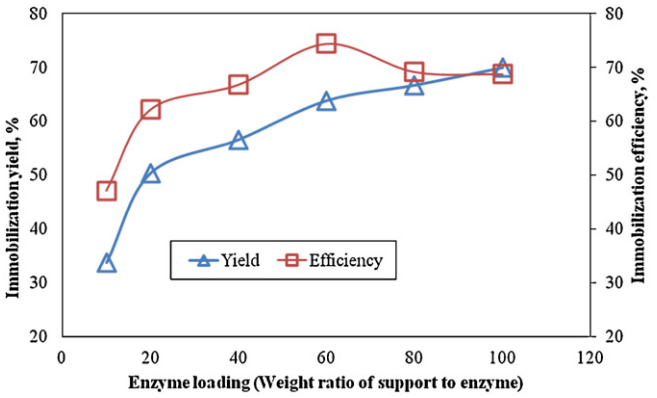

Fig. 1. The effects of enzyme loading on immobilization of PheDH from *Rhodococcus* sp. strain M4 onto Eupergit CM.

2.4.4. Synthesis of (S)-2-amino-4-phenylbutanoic acid

Asymmetric synthesis of S-APBA was carried out in batches by adding the immobilized PheDH in 10 mL of reaction mixture containing 10 mM OPBA, 5 mM NADH, ammonium formate, formate dehydrogenase in 50 mM pH 8.5 Tris–HCl buffer and incubated at 30 °C for 24 h. Aliquots of the reaction mixtures were drawn out to determine conversion of the substrate using HPLC (Agilent 1200 Series) on a ZORBAX Eclipse XDB-C18 column (Agilent Technologies) with gradient elution from an initial solution of 90% of eluent A + 10% of eluent B to a final solution of 100% of eluent B in 20 min, at 1 mL/min flowrate with detection at 214 nm. Eluent A consists of 5% acetonitrile in 0.1% trifluoroacetic acid and eluent B consists of 95% acetonitrile in 0.1% trifluoroacetic acid.

2.4.5. Determination of the enatiomeric excess of (S)-2-amino-4-phenylbutanoic acid

The enantiomeric excess of the synthesized S-APBA was determined using chiral HPLC on an Astec CHIROBIOTIC T column (Orbiting Scientific Co.) using ethanol/water, 10/90 (v/v) as mobile phase. The flow rate was set at 1 mL/min with detection at 210 nm.

3. Results and discussion

3.1. Effect of the immobilization paramaters

Some common immobilization parameters that are widely studied to evaluate the immobilization performance are enzyme loading (enzyme/carrier weight ratio), pH and ionic strength of the immobilization buffer solution, temperature and incubation time [17,21–23].

Fig. 1 shows the effects of the enzyme loading on the immobilization in the range between 10 and 100 mg of support to 1 mg of enzyme. The immobilization yield increased with the increasing ratio, whereas the immobilization efficiency decreased. A sharp increase of total protein bound was observed at the 20:1 ratio (w/w) of support to enzyme, with no significant increase in immobilization yield obtained with further increase of support weight. This observation concurs with findings by several reported works by others in which the immobilization efficiency of enzymes on solid support decreases upon reaching a saturation value [22,24–26]. At the weight ratio of 60:1, immobilization of PheDH onto Eupergit CM yielded the highest immobilization efficiency (74.4%) and a high immobilization yield (63.8%). Despite the higher immobilization yield at the higher weight ratio (80:1 and 100:1), the immobilization efficiency declined, which could be attributed to steric hindrance that prevented the substrate accessibility to the immobilized enzyme [13]. A more extensive multi covalent linkages between the enzyme and the supports may have been formed and disturbed the globular structure of the enzyme; hence affecting the enzyme catalytic capability [27,28].

Table 1
The effects of pH and molarity of buffer solution on immobilization of PheDH from *Rhodococcus* sp. strain M4 onto Eupergit CM.

pH	Molarity mM	Immobilization yield (%)	Immobilization efficiency (%)
4.0	10	32.9	–
	100	42.7	–
	500	60.1	–
	1000	66.5	–
7.0	10	33.2	26.3
	100	50.7	26.9
	500	61.1	48.1
	1000	68.0	16.1
8.5	10	33.9	39.7
	100	50.8	59.1
	500	62.9	74.4
	1000	70.2	52.8
10	10	32.9	38.4
	100	49.8	53.6
	500	61.7	69.1
	1000	67.1	48.9

Investigations were carried out to study the effect of the pH on the immobilization yield and efficiency. In agreement with a common observation reported by several researchers [22,29], there were no significant variances of immobilization yield over the pH range from acidic (pH 4.0), neutral (pH 7.0) to alkaline (pH 8.5) conditions. The epoxy functional groups on Eupergit could react with different nucleophile groups of the enzyme over a wide range of pH values. However, deterioration of immobilization efficiency was observed at acidic condition, as could be seen from Table 1. This observation concurs with the results shown in Section 3.2.1, in which it was found that PheDH displayed low enzyme activity and stability at acidic condition.

Besides the pH of the immobilization buffer, it is widely reported that immobilization onto oxirane functionalized supports are strongly affected by ionic strength of the immobilization buffer. A high ionic strength of the immobilization buffer increases the strength of hydrophobic interactions between enzyme and Eupergit; hence promoting and accelerating the immobilization process [30]. Knezevic et al. [31] reported that although utilization of a high ionic strength buffer was necessary to promote the covalent immobilization onto Eupergit supports by conventional method [29], some enzymes could not withstand such conditions. Increment of 12.1% in immobilization yield was observed for immobilization carried out in buffer pH 8.5 of 500 mM when compared to the same buffer of 100 mM. Despite the high immobilization yield achieved by immobilization in 1000 mM buffer, the catalytic properties of the immobilized enzyme deteriorated, probably due to conformation and structure distortion of the enzyme.

The incubation time notably influenced the PheDH immobilization yield (Fig. 2). A longer incubation time yielded a higher immobilization yield and immobilization efficiency. Beyond the period of 24 h, the increment of immobilization yield was relatively slow, at the rate of 0.5% for every 12 h. However, the increment of immobilization efficiency at a higher rate of 1.2% suggested that a longer incubation time might result in induction of increased multipoint attachment [32].

Through performance evaluation of the immobilization based on the yield and the efficiency, an adequate combination of the immobilization conditions are determined to obtain optimum values in these parameters. The subsequent experiments and analysis were carried out using PheDH immobilized onto Eupergit CM prepared at enzyme loading of 60:1 weight ratio of enzyme

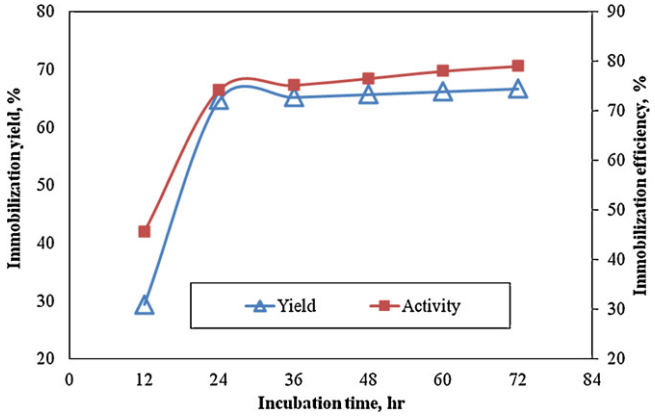

Fig. 2. The effects of incubation time on immobilization of PheDH from *Rhodococcus* sp. strain M4 onto Eupergit CM.

carrier to enzyme in an immobilization buffer of pH 8.5 at 500 mM for 24 h.

3.2. Characterization of free and immobilized PheDH

3.2.1. Effect of the pH on enzyme stability and activity

In enzyme stability study, enzyme was preincubated in 10 mM buffer at different pH values ranging from 4 to 11 for 30 min at 25 °C prior to the activity assay. A moderate improvement in pH dependence was achieved by the immobilized PheDH, showing broader maxima from pH 6 to pH 11 as opposed to the free enzyme with pH range from pH 6 to pH 9.5, as shown in Fig. 3. However, both enzymes displayed a similar optimum pH 8.5 [19]. Preincubation

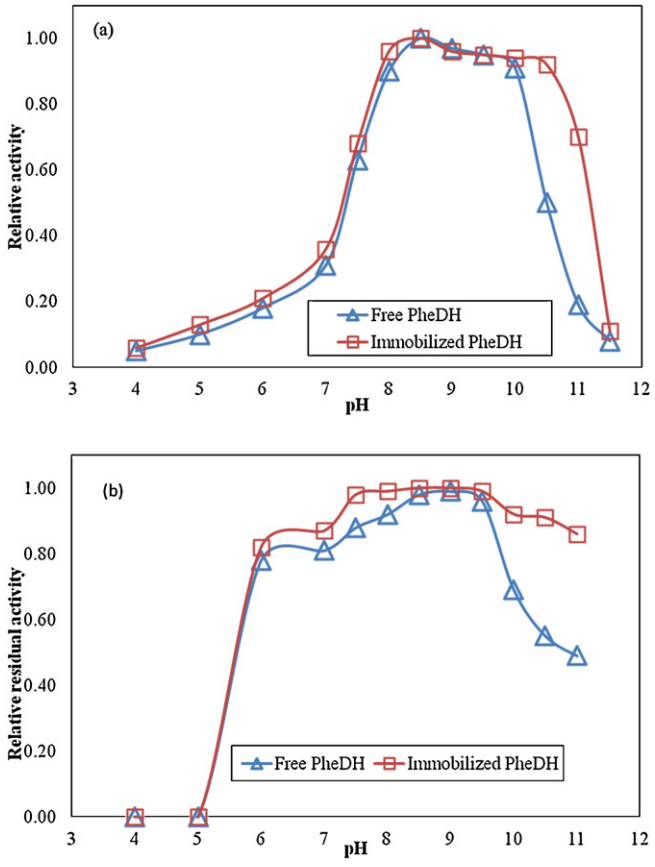

Fig. 3. The effects of pH on (a) enzyme activity and (b) stability.

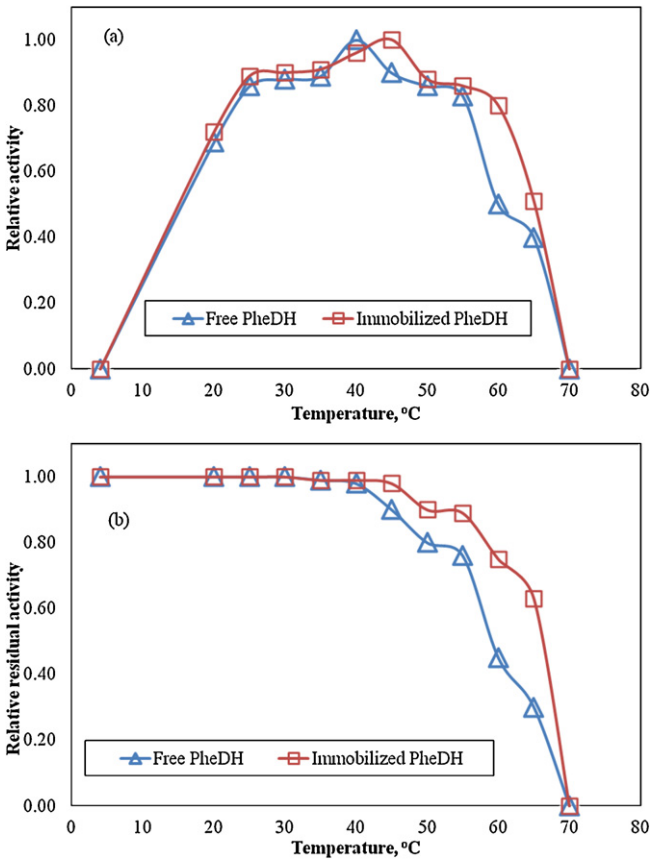

Fig. 4. The effects of temperature on (a) enzyme activity and (b) stability.

of PheDH between pH 6 and pH 9.5 did not show significant effect on the enzyme activity, whereas significant decline of the enzyme activity was observed for pH lower than 6 and higher than 9.5. Deterioration of enzyme activity may result from formation of improper ionic form of the enzyme and/or substrate [33].

A notable difference of pH dependency for immobilized and free enzyme was observed at a narrow pH range of 7.5–9.5, where the former showed an almost constant activity. This observation was similar to works reported by Alptekin et al. [32], where it was believed that covalent bonding of enzyme with oxirane groups on Eupergit may result in physicochemically stable enzyme, stabilizing the pH of the microenvironment surrounding the enzyme. The hydrophobicity of Eupergit may also reduce the dielectric constant of the microenvironment with consequent modification of the acidity constants of acidic and basic groups on the immobilized enzyme.

3.2.2. Effect of the temperature on enzyme stability and activity

Fig. 4(b) shows the thermostability profile of the enzymes at various temperatures which are notably similar. The optimum temperature for the immobilized PheDH improved to 50 °C from 40 °C for the free PheDH as can been seen from Fig. 4(a). The enzyme activity increases as the temperature increases to its optimum value. The free enzyme displayed a lower relative activity throughout the profile.

3.2.3. Reusability and storage stability of PheDH

In the reusability study of the immobilized PheDH, reaction cycle was fixed at 1 h for each batch. The residual activity of the enzyme was determined for every reaction cycle, after rinsing the recovered enzyme with Tris–HCl buffer of 50 mM at pH 8.5. A remarkable improvement in reusability of the immobilized PheDH

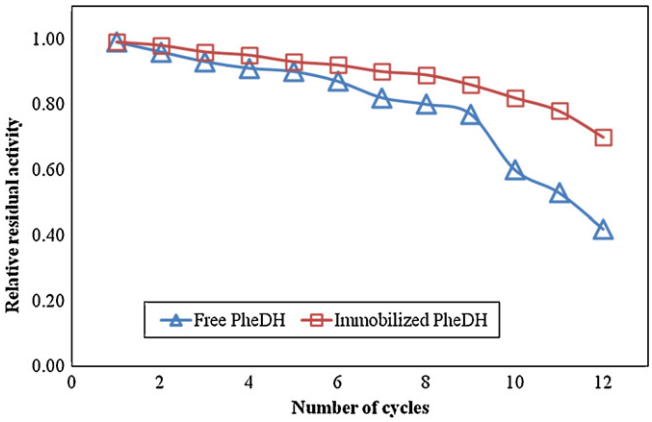

Fig. 5. Reusability of free and immobilized enzymes.

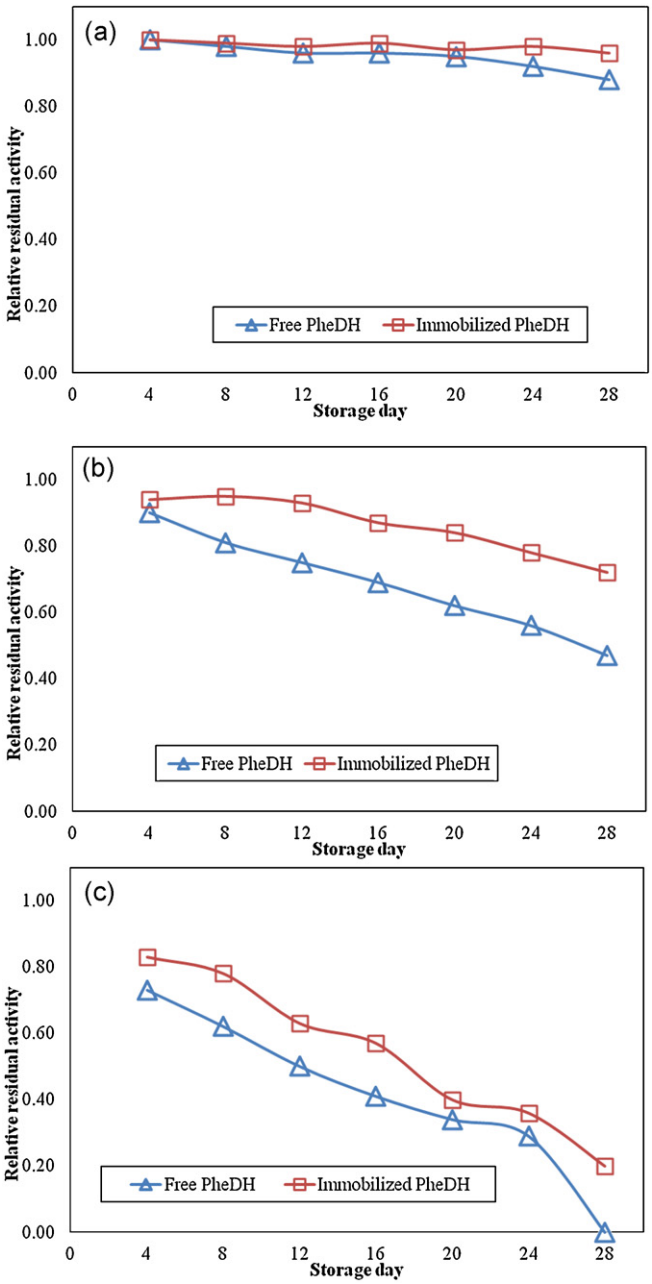

Fig. 6. Storage stability of free and immobilized enzymes at (a) freezer temperature (−20 °C); (b) refrigerator temperature (4 °C) and room temperature (25 °C).

was obtained, at 9 cycles with less than 15% activity decline, in comparison to the free PheDH which could only be reused for 4 cycles (Fig. 5).

As for the storage stability study, the enzymes were stored in 51% wt/wt glycerol at room temperature (25 °C), refrigerator temperature (4 °C) and freezer temperature (−20 °C). The residual activities were measured over a course of 28 days of storage time. Fig. 6 shows the storage stability profiles for both enzymes. Both enzymes were not stable at room temperature with rapid deactivation observed. Eventually, the residual activity detected for the immobilized enzyme was merely 20% at day 28, whereas the free enzyme was completely deactivated. Immobilization onto epoxy-functionalized support seemed to improve the shelf life of the enzyme as the formed covalent bonds could enhance the conformational stability of the derivates [32]. Although both enzymes showed gradual deactivation in cold storages, the residual activity of immobilized enzyme remains high at the end of the study, at the value of 72% in comparison to free enzyme (47%) for storage at 4 °C. Storage at −20 °C is favorable for both enzymes as the deactivation is kept at minimal level, with detectable high residual activity of 96% for the immobilized PheDH and 82% for the free enzyme.

3.3. Application of immobilized PheDH for the synthesis of S-APBA

The immobilized PheDH was used as biocatalyst for the asymmetric synthesis of S-APBA, carried out in batches. From the HPLC analysis using a C18 column for the aliquot of the reaction mixture, it was observed that the substrate was converted into product with a yield of more than 80%. The enantiomeric excess of S-APBA obtained was more than 99% as proven by the HPLC analysis using a Chiral T column where only a single peak was eluted. These observations are similar to our previously reported work [9]. Additionally, there was no leaching of bound PheDH from Eupergit CM, as monitored spectrophotometrically. No enzyme activity was detected in the aliquot of the reaction mixture.

4. Conclusions

PheDH enzyme from *Rhodoccocus sp.* 4 was immobilized onto Eupergit CM and tested for the synthesis of S-APBA. Studies on the effects of various immobilization parameters showed that the immobilization of PheDH is best to be carried out at enzyme loading of 60:1 (weight ratio of enzyme carrier to enzyme) in an immobilization buffer of 500 mM at pH 8.5 for 24 h. Upon covalent immobilization onto Eupergit CM, improvements on the operational pH range, the thermostability, the operability and storage stability were achieved. The immobilized PheDH was successfully applied for the synthesis of (S)-2-amino-4-phenylbutyric acid, achieving enantiomeric excess of more than 99% and yield of more than 80%; comparable to the synthesis using the free PheDH. Reductive amination using the immobilized PheDH is beneficial to enhance asymmetric synthesis S-APBA.

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