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ORIGINAL RESEARCH PAPER

The Immobilization of *Candida antarctica lipase* B by ZIF-8 encapsulation and macroporous resin adsorption: preparation and characterizations

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Abstract A novel enzyme immobilization method employing metal–organic framework (MOF) encapsulation and macroporous resin adsorption was developed in this study. *Candida antarctica* lipase B (CALB) was firstly encapsulated onto metal–organic frame structures (Zeolitic imidazole framework-8, ZIF-8) and further bonded to macroporous resin by physical adsorption. Under optimized immobilization conditions, the activity of the prepared immobilized lipase (CALB-ZIF-8@D101) determined via the methyl esterification of oleic acid was 38.4 U/mg. Compared with free lipase, the immobilized lipase exhibited improved thermal and operational stability and organic solvent tolerance. These results demonstrate that the immobilization method of ZIF-8 encapsulation and macroporous resin adsorption enhanced enzyme properties at a superior level.

Keywords Immobilization · *Candida antarctica* lipase B · Zeolitic imidazole framework · Macroporous resin · Characterization

Introduction

Lipases (EC.3.1.1.3, triacylglycerol acyl hydrolase) have always been the focus of biotechnology, thanks to the functions of these biocatalysts, which can provide highly efficient catalytic hydrolysis, transesterification and esterification under environmentally friendly conditions (Stergiou et al. 2013; Salihu and Alam 2015; Navvabi et al. 2018). Nonetheless, numerous problems exist in the practical applications of free enzymes, including exorbitant expenditure, recovery difficulties, thermal instability and poor organic solvent tolerance. Fortunately, enzyme immobilization could be the focal point towards improving enzyme properties (catalytic activity, solvent tolerance, thermal stability and recyclability) (Liu et al. 2011; Arana-Pena et al. 2019).

Immobilized lipase is an excellent biocatalyst widely employed in industrial applications. Enzyme immobilization aims to improve lipase's economic efficiency and reusability (Zhou et al. 2018; Lee et al. 2019). As outstanding enzyme-fixing carriers, metal–organic frameworks (MOFs) can effectively increase enzyme stability and recycling rates (Wu et al. 2017;

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Pan et al. 2011). MOFs have remarkable potential in industrial applications through their improvement of various enzyme properties (Liang et al. 2015; Urrutia et al. 2018). Problems such as enzyme leakage and insufficient substrate-enzyme interactions when they are used continuously still need to be resolved (Mehta et al. 2016; Rodrigues et al. 2019).

We utilized ZIF-8 encapsulation and macroporous resin adsorption to deal effectively with this issue. In this study, *Candida antarctica* lipase B (CALB) was firstly encapsulated onto metal–organic ZIF-8 frame structures (Jung and Park 2016), then combined with macroporous resin through physical adsorption adding polyethyleneimine (as shown in Fig. 1). Characterizing the formation mechanism and internal structure of the immobilized lipase by scanning electron microscope (SEM), dynamic light scattering (DLS), X-ray diffraction (XRD) and thermal gravity (TG) was discussed, as well as the parameters for enzyme immobilization (the preparation time and pH, the molar ratio of dimethylimidazole to zinc nitrate, the amount of added CALB and the macroporous resin type) (Jiang et al. 2019; Enayati et al. 2018). Moreover, the solvent tolerances, storage stability, and thermal and operational stabilities of immobilized lipase were also investigated (Hou and Ge 2017; Zhao et al. 2017).

Materials and methods

Materials

Lipase CALB (*Candida antarctic* lipase B, 500 LU/g hydrolytic activities) was purchased from Novozymes, Denmark. Resin D101 was purchased from Zhengzhou Qinshi Technology Co., Ltd., while Resins HPD850 and HPD950 were obtained from Sepdex Co. Resin H103 was from Jiangsu Jinkai Resin Chemical Co., Ltd.; Resin XAD1180N was from Sigma-Aldrich Co.; Resins XAD1600N and XAD7HP were from Rohm and Haas Co.; and Resin D3520 was from Tianjin Nankai Hecheng Technology Co., Ltd. Polyethyleneimine (PEI) was from Shanghai Aladdin Co. All other chemicals were of analytical grade and utilized without further purification. Deionized water was used throughout the experiments.

Immobilization procedures

ZIF-8 encapsulation of CALB

0.3125 mol/L of dimethylimidazole solution and 0.3125 mol/L of zinc nitrate solution were subjected to ultrasonic treatment for 20 min (Nadar and Rathod 2018). Approximately 60 mL of dimethylimidazole solution and 1.5 mL of zinc nitrate solution were added to a 250 mL Erlenmeyer flask, after which 3 mL of CALB was slowly added to the flask (Chu et al. 2019; Jung and Park 2016). The resulting

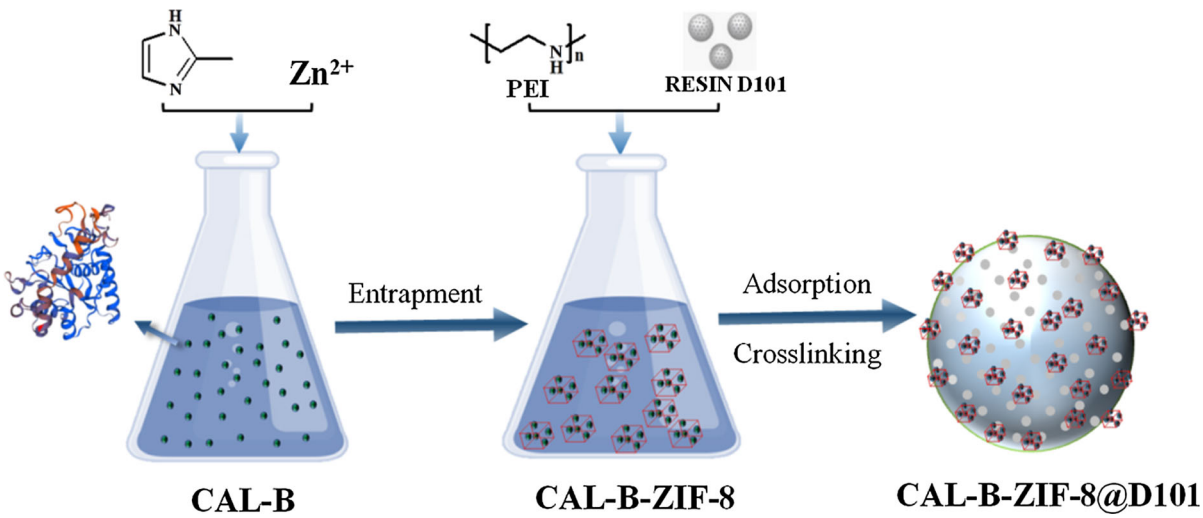

Fig. 1 Schematic diagram of the preparation procedures of CALB-ZIF-8@D101

solution was shaken at 25 °C and mixed at 200 rpm with a magnetic stirring method for 30 min. The MOF enzyme complex (CALB-ZIF-8) was obtained by encapsulation of the enzyme molecule in ZIF-8 (Bui et al. 2018; Mao et al. 2014; Lyu et al. 2014).

Macroporous resin adsorption of CALB-ZIF-8

2.5 mL of a cross-linking agent (25% PEI) and 0.5 g of macroporous resin were sequentially added to the CALB-ZIF-8 complex. The resulting solution was shaken for 5 h. at 30 °C and 200 rpm in a water bath shaker. CALB-ZIF-8@D101 was obtained by suction filtration, then dried in a vacuum oven at 35 °C for 4 h and stored at 4 °C.

Esterification activity of immobilized lipase

Reaction system 200 mg of immobilized lipase CALB-ZIF-8@D101 was added into a mixture of oleic acid (1 mL, 3.15 mmol), tert-butanol (0.5 mL, 5.23 mmol), methanol (0.5 mL, 12.36 mmol) and deionized water (40 μL) in a 2.0 mL EP tube. The reaction mixture was shaken in a water bath of 200 rpm and 35 °C for 1 h (Cea et al. 2019). Subsequently, 10 μL of the reaction solution was added to 990 μL of ethyl acetate for gas chromatography (GC) analysis. The production of methyl oleate in the reaction system was determined.

Gas chromatographic conditions Agilent 6890 gas chromatograph, DB-23 capillary column (60.0 m × 0.32 mm × 0.25 μm), FID detector: detection conditions were at the column oven temperature of 220 °C, at constant temperature, for 10 min; the injection port temperature was 250 °C, the detector temperature was 250 °C, the carrier gas was high purity nitrogen and the split ratio was 20:1.

Structural characterization of CALB-ZIF-8@D101

The structural characterization of CALB-ZIF-8 and CALB-ZIF-8@D101 were studied using a dynamic light scattering instrument (DLS Nanobrook omni, Brookhaven Instruments Corporation, USA), X-ray diffraction (XRD X' Pert PRO, PNAlytical, Netherlands), scanning electron microscopy (SEM S-4800, Hitachi Limited, Japan) and thermal gravity (TG TA Q500, TA Instruments, USA). The binding structure and enzyme content of the immobilized enzyme were determined.

Thermal stability of CALB-ZIF-8@D101

The thermal stability of CALB-ZIF-8@D101 was measured by determining the residual esterification activities after incubation in tert-butanol at 50 °C, 60 °C and 70 °C over a period of time (Yang et al. 2019; Cai et al. 2018). The remaining activities of the immobilized lipases were measured as described in Sect. 2.3. The relative activity of CALB-ZIF-8@D101 prior to incubation was defined as 100%.

Operational stability of CALB-ZIF-8@D101

In order to evaluate the operational stability of immobilized lipase at repeated use, the reusability of CALB-ZIF-8@D101 in the methyl esterification of oleic acid was determined. The remaining activities of the immobilized lipases were measured as described in Sect. 2.3. The reaction system was filtered through filter paper, and the recovered immobilized enzyme was washed twice with tert-butanol and then reused in the next batch of reactions. The enzyme activity for each batch was calculated as a comparison.

Organic solvent tolerance of CALB-ZIF-8@D101

To investigate the organic solvent tolerance of free CALB, CALB-ZIF-8 and CALB-ZIF-8@D101 were reacted in organic solvent (tert-butanol, n-hexane, toluene, DMSO and DMF). The activities of the immobilized lipase were measured as described in Sect. 2.3.

Results and discussion

Effects of immobilization parameters

MOF encapsulation process

The effects of the reaction time, pH and molar ratio of dimethylimidazole to zinc nitrate and the addition amount of enzyme were taken into consideration in the current optimization process. The reaction time was from 10 to 60 min, and the MOFs' highest yield and vigor were obtained following a 30 min reaction

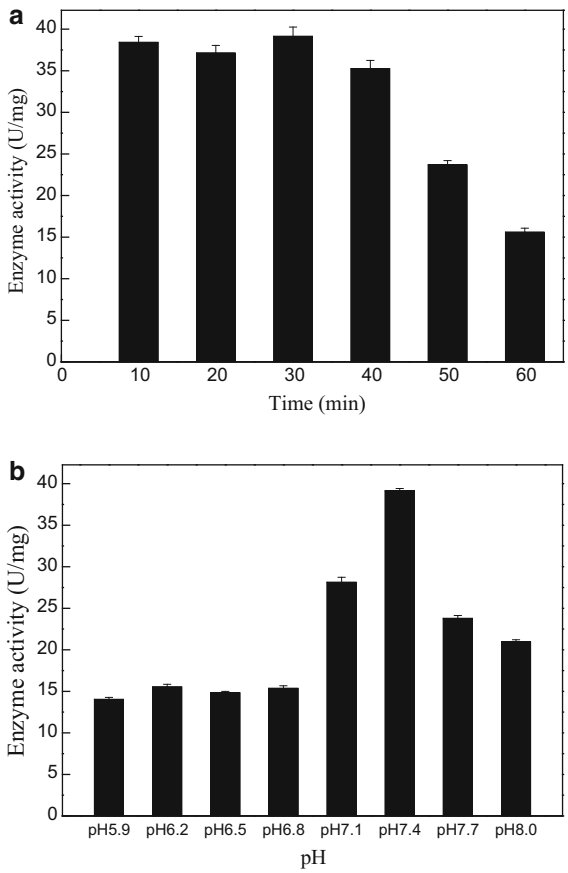

Fig. 2 Effect of the reaction time (**a**) and the pH value (**b**) on the activity of immobilized lipase

(Fig. 2a). During the preparation of the MOFS, buffers of different pH were used for washing, and the most viable ZIF-8 was obtained under the conditions of the pH 7.4 buffer (Fig. 2b). As the molar ratio of dimethylimidazole to zinc nitrate was increased from 25:1 to 40:1, the immobilized enzyme activity also increased. However, further increases in molar ratio from 40:1 to 60:1 resulted in a decrease in the immobilized enzyme activity (Fig. S1a). As the addition amount of enzyme raised from 70 to 210 mg, the immobilized enzyme activity likewise increased. However, excessive enzyme had the potential of leading to mineralization failure (Fig. S1b).

Resin adsorption process

Macroporous resin is a cross-linked polymer which has a relatively large specific surface and an appropriate pore size. Physical and chemical properties vary from resin to resin. We screened seven macroporous resins (D101, XAD1600N, HPD850, XAD1180N, XAD7HP, D3520, and H103) and found that the D101 resin had the best adsorption capacity (Fig. S2a). The resin amount in the 60 mL system ranged from 0.25 to 2.00 g. As the resin concentration increased, both the amount of enzyme attached per unit of resin and the activity of the immobilized lipase decreased. Immobilized lipase showed highest activity when the amount of resin additive was 0.5 g (Fig. S2b). Therefore, Resin D101 with 0.5 g resin added was the optimal choice.

The characterization of immobilized lipase

DLS and XRD analysis of immobilized lipase

The characterization of the particle size and crystal form of the ZIF-8 and CALB-ZIF-8 obtained were investigated in detail by DLS and XRD. According to the test results from dynamic light scattering, the particle size of ZIF-8 and CALB-ZIF-8 reached 9421 ± 102 and $18{,}594 \pm 214$ nm, respectively (Fig. S3). As shown in Fig. S3b, the CALB-ZIF-8's particle size was significantly changed with respect to ZIF-8. As shown from Fig. S4, the CALB-ZIF-8 crystal structure changed compared to ZIF-8. Thus, this method was able to tightly bind the enzyme protein to ZIF-8.

TG analysis of immobilized lipase

To investigate the quantity of enzyme protein in CALB-ZIF-8, the thermal degradation process of CALB-ZIF-8 was analyzed with the TG method in an aerial atmosphere. As shown in Fig. S5, the detection temperature was within the range of 0–600 °C, and the heating rate was 20 °C/min. The hybrid composite displayed a sharp weight decrease in the temperature range of 300–500 °C, which compared with ZIF-8 in the absence of the entrapped enzyme. The net weight change induced by enzyme decomposition was observed to be 10.6%.

SEM analysis of immobilized lipase

The surface characterizations of the immobilized enzymes CALB-ZIF-8 and CALB-ZIF-8@D101 was analyzed by SEM. There is a large difference between

the CALB-ZIF-8 and CALB-ZIF-8@D101 surface characteristics, according to their SEM micrographs performed at a magnification of 2×10^4 (Fig. S6). The rigid structure of ZIF-8 provided a discrete space capable of reducing the overlap of enzymes molecules, thereby incrementing not only enzyme activity but also enzyme temperature and solvent tolerance. The CALB-ZIF-8 was adsorbed and cross-linked onto Resin D101, and also resolved the problem of isolating CALB-ZIF-8 from the reaction substrate. The larger specific area of the resin was capable of providing a greater space for substrate contact and reducing steric hindrance, which was beneficial to the progress of the catalytic reaction.

The kinetics analysis of the immobilized lipase

The kinetic analysis of CALB-ZIF-8@D101 and free enzyme (apparent Michaelis constant *K*m) for the transesterification between oleic acid and methanol with CALB-ZIF-8@D101 was calculated with the Lineweaver–Burkplot method. The *K*m and *V*max of CALB-ZIF-8@D101 and the free enzyme were 0.8662 mM; 33.01 mmol/min; and 0.7455 mM, 69.93 mmol/min, respectively (Fig. S7). The kinetic constant indicated that CALB-ZIF-8@D101 had a good catalytic effect on the transesterification between oleic acid and methanol. The free enzyme was more active but was susceptible to inactivation during the reaction. In view of the relatively small amount of enzyme protein attached to CALB-ZIF-8@D101, the method also improved the properties of the enzyme.

The stability of immobilized lipase

Thermal stability

The thermal stability of CALB-ZIF-8@D101 and CALB-ZIF-8 was superior to that of the free lipase. It was likely that the rigid ZIF-8 structure limited the structural rearrangement at high temperatures (Liang et al. 2016; Pan et al. 2011). In this study, thermostability was examined by incubating immobilized enzymes in tert-butanol at different temperatures (50 °C, 60 °C, and 70 °C) for different periods of time. CALB-ZIF-8@D101 retained high residual activity as the temperature increased. The thermal stability of the free and immobilized enzyme was investigated in tert-butanol at 70 °C (Fig. 3). The

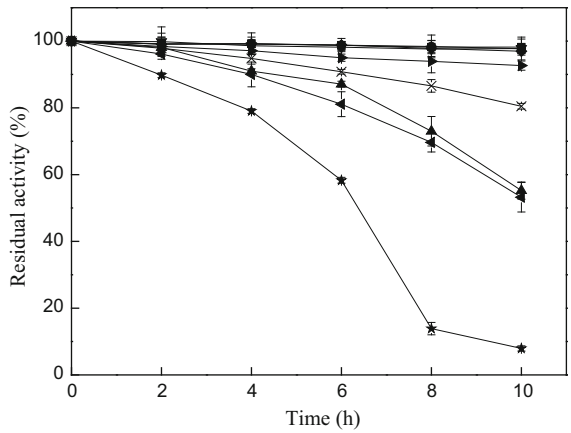

Fig. 3 Thermal stability analysis of CALB-ZIF-8@D101 at 50 °C (filled square), 60 °C (filled circle), 70 °C (filled triangle) and CALB-ZIF-8 at 50 °C (inverted filled triangle), 60 °C (filled diamond), 70 °C (left pointed filled triangle) free enzyme at 50 °C (right pointed filled triangle), 60 °C (times symbol), 70 °C (star)

results showed that activity rate of the free enzyme, CALB, was 7.93% of its original activity following incubation for 10 h at 70 °C, while CALB-ZIF-8@D101 retained 56.21% of its initial activity.

Solvent tolerance stability

The effects of different organic solvents (tert-butanol, n-hexane, toluene, DMF and DMSO) on the activity of immobilized and free enzymes were investigated. CALB-ZIF-8 @D101 and CALB-ZIF-8 exhibited higher tolerance in organic solvents than in free lipase

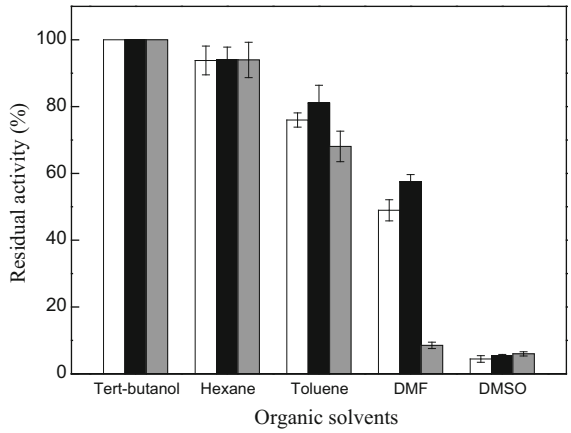

Fig. 4 Residual activity of CALB-ZIF-8@ D101 (white), CALB-ZIF-8(black) and free enzyme (gray) in tert-butanol, n-hexane, toluene, DMF and DMSO

(Fig. 4) (Wu et al. 2017). This stronger degree of solvent tolerance can be attributed to the rigid ZIF-8 structure limiting the groups' structural rearrangement. Moreover, using ZIF-8 for encapsulation greatly improved the solvent resistance of the enzyme and, therefore, had good industrial value (Wu et al. 2015).

Operational stability

The reusability of CALB-ZIF-8 @D101, CALB-ZIF-8 and free enzyme was measured by repeated catalytic esterification. After each cycle, the biocatalyst was recovered by centrifugation and washed with tert-butanol prior to the next reaction to remove the unreacted substrate and product (Sola-Rabada et al. 2018; Lian et al. 2018). As shown in Fig. 5, it was found that CALB-ZIF-8 @ D101 can be used continuously for 10 cycles with an activity loss of 15.8%. The free enzyme experienced a more significant loss with a residual enzyme activity of 31.61%. CALB-ZIF-8 as a powdery crystalline substance has a good effect on improving the stability of the enzyme, while the resin reduced the mobility of the enzyme.

Storage stability

The immobilized enzymes were placed at 4 °C and 25 °C, and their enzyme activity was measured every 5 days. Both activity rates remained high at 4 °C, and at room temperature the free enzyme activity greatly decreased, The enzyme activities of CALB-ZIF-8@D101 decreased more slowly, at 94.41% in35 days. This indicates that the immobilized enzyme was more stable than the free enzyme during long-term storage (Fig. S8).The CALB-ZIF-8 @D101 was stored under different pH conditions for 2 to 10 h and subjected to methyl esterification. The immobilized enzyme was found to have excellent pH stability (Fig. S9).

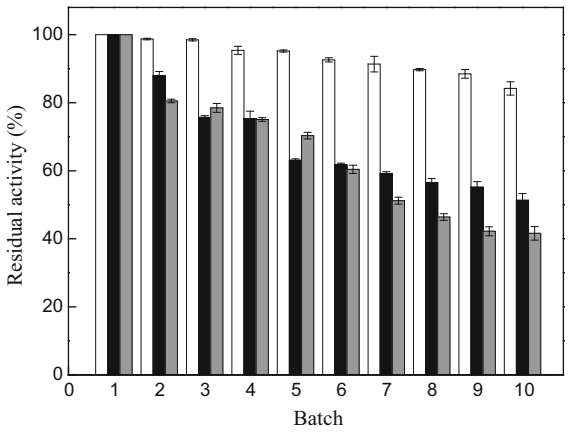

Fig. 5 Operational stability of CALB-ZIF-8@D101 (white), CALB-ZIF-8(black) and free enzyme (gray)

Conclusions

We have successfully established a new, two-step, lipase immobilization of CALB, which combines ZIF-8 encapsulation with resin adsorption. Due to the homogeneous distribution of cavities produced throughout the entire sample by ZIF-8, the enzyme is unable to further aggregate in solution. The rigid ZIF-8 structure limits the structural rearrangement that leads to denaturation. Compared to free CALB, CALB-ZIF-8@D101 exhibits superior thermal stability, organic solvent resistance and handling stability. Especially in DMF, CALB-ZIF-8@D101 exhibits extraordinary solvent resistance. Moreover, it exhibits an excellent reusability in the synthesis of methyl oleate. After 10 cycles, 84.20% of the initial activity has been maintained. In conclusion, this lipase's immobilization has promising developmental possibilities for industrial applications.

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Supporting information Supplementary Figure S1—Effect of the molar ratio (a) and the addition amount of enzyme (b) on the activity of immobilized lipase.

Supplementary Figure S2—Effect of the resin types (a) and the resin addtion (b) on the activity of immobilized lipase.

Supplementary Figure S3—DLS parameter of ZIF-8 (a) and CALB-ZIF-8 (b).

Supplementary Figure S4—XRD patterns of ZIF-8 (a) and CALB-ZIF-8 (b).

Supplementary Figure S5—TG analysis of ZIF-8 (red) and CALB-ZIF-8 (black).

Supplementary Figure S6—SEM images of ZIF-8 (a) and CALB-ZIF-8@D101 (b).

Supplementary Figure S7—Lineweaver-burk plots of CALB-ZIF-8@D101 (a) and free enzyme (b).

Supplementary Figure S8—Storage stability of CALB-ZIF-8@D101 at 4 °C (fiiled square), 25 °C (filled circle) and free enzyme at 4 °C (filled triangle), 25 °C (filled inverted triangle).

Supplementary Figure S9—pH storage stability of CALB-ZIF-8@D101 for pH6.0 (fiiled square), pH6.5 (filled circle), pH7.0 (filled triangle, pH7.5 (filled inverted triangle), pH8.0 (filled diamond).

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