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Immobilization of *Candida antarctica* lipase B onto SBA-15 and their application in glycerolysis for diacylglycerols synthesis

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ABSTRACT

In this study, *Candida antarctica* lipase B (CALB) was immobilized on SBA-15 with three pore diameters. CALB loading was found increased with CALB concentration increasing from 20.3 to 80.12 μg/ml. Higher CALB loading was observed from SBA-15 with pore diameters at 8.1 nm (SBA-15(8.1)), yet highest hydrolytic activity was found at SBA-15(12.5). Thermal stability of the immobilized CALB (SBA-15-CALB) samples was greatly influenced by their water content, after 4 h storage at 70 °C, 8.93 and 67.4% of the initial activity was observed from SBA-15-CALB samples with water content at 9.23 and 3.22% respectively. The SBA-15-CALB samples were then used in glycerolysis of corn oil, and 53.6 wt% of diacylglycerols was obtained after optimization. The operational stability was tested, and after 5 consecutive applications, 92.5 and 80.3% of the initial glycerolysis activity was remained respectively from SBA-15(6.6)-CALB and SBA-15(12.5)-CALB.

1. Introduction

Immobilization of lipase facilitates the separation of products and the recovery of lipase for reuse. In addition, immobilization also confers advantageous features including enhanced activity, improved stability and increased selectivity (Chen, Miller, Miller, Maikner, & Gross, 2007; Forde et al., 2010). Generally, lipase can be immobilized through crosslinking, covalent attachment, or physical adsorption (Idris & Bukhari, 2012). Of which, physisorption may have higher commercial potential due to it simpleness low-cost as well as retaining high lipase activity (Gao, Wang, Wang, Luo, & Dai, 2009; He, Song, Ma, Yang, & Guo, 2006). Nevertheless, the stability of the physically adsorbed lipase tends to be poor, because adsorption is generally a relative weak interaction and the lipase will desorb from support during reaction and washing (Gao, Wang, Diao, Luo, & Dai, 2010; Zheng et al., 2012). Covalent attachment can remedy the leaching disadvantage but it may significantly reduce the activity of lipase.

Carriers play an important part in the lipase immobilization. Leaching of lipase can be diminished without being covalently bonded to the wall, when ordered mesoporous materials (OMM) were used as the supports (Serra, Mayoral, Sakamoto, Blanco, & Díaz, 2008). OMM are potential candidate carriers, due to their advantageous features, including high surface areas, good connectivity, big tortuosity as well as pore size tunability. SBA-15 is the most representative example, its average pore diameter is usually around 8 nm, which makes it an ideal candidate for the immobilization of lipase.

CALB is a highly versatile enzyme with appromximate dimensions of $3 \times 4 \times 5$ nm and molecular weight of 32 kDa, it can catalyze a variety of reactions including kinetic resolutions, aminolysis, esterification, transesterification and hydrolysis (Idris & Bukhari, 2012). In addition, CALB is also organic solvents resistant, reasonable thermal stable, stereospecific and enantiospecific (Forde et al., 2010). CALB in immobilized forms offer some operational advantages over their soluble counterparts in practical applications. Despite the already existence of commercially available Novozym 435, studies regarding CALB immobilization are continued at a rapid rate. The properties of the immobilized lipase were influenced greatly by the immobilization technique, immobilization conditions, nature of solvent and variety of reactor. Therefore, the properties of lipase for a particular application can be theoretically tailored through immobilization (Idris & Bukhari, 2012).

Immobilization of CALB on SBA-15 has been studied by some authors. Of which, Abdallah et al. used SBA-15 and a porous spherical silicate material (PPS) to immobilize CALB, they found that CALB exhibited higher activity and stability on SBA-15 than on PPS, due to the different physical properties of the SBA-15 and

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PPS (Abdallah et al., 2014). Laszlo et al. investigated the effects of matrix morphology and surface polarity on the CALB immobilization, and SBA-15 was found to enable to protect the immobilized CALB from denaturation and the all absorbed CALB was active (Laszlo, Jackson, & Blanco, 2011). Forde et al. employed three different types of mesoporous silicate to immobilize CALB, results illustrated that SBA-15 was able to create a stable environment for lipase and prevent damage by external shear forces. Approximately 30-fold stability improvement of the chmical modified CALB was observed after immobilization on SBA-15 (Forde et al., 2010). All these advances make SBA-15 potential candidate for CALB immobilization. However, systematic study on the immobilization of CALB onto SBA-15 has not been seen. In addition, applications of the SBA-15 supported CALB (SBA-15-CALB) as biocatalysts were still limited, catalytic performance of the SBA-15-CALB was largely evaluated by activity test through simple reactions, like hydrolysis of tributyrin or triacetin, some other important reactions have been rarely tested.

With its high time-space cost efficiency, glycerolysis of triacylglycerols (TAG) is quite important in lipid modification field (Huang, Gao, & Zhong, 2015). It is the primary reaction route for the production of monoacylglycerols (MAG) and diacylglycerols (DAG). MAG and DAG are widely used in food, pharmaceutical and cosmetic industries (Krüger et al., 2010). MAG are the most important food-grade emulsifiers, accounting for about 75% of the worldwide production of emulsifiers in food industry (Guo & Xu, 2005). DAG, especially 1,3-DAG, have been claimed to be capable of preventing body fat accumulation. DAG-enriched oil has been recognized as a functional cooking oil, which has gained tremendous interest as a functional food to replace the conventional TAG oil for obesity management (Phuah et al., 2015).

In this study, CALB was immobilized onto SBA-15 with three different pore diameters, 6.6, 8.1 and 12.5 nm (labelled as SBA-15 (6.6, 8.1 and 12.5) before and SBA-15(6.6, 8.1 and 12.5)-CALB after CALB immobilization respectively). Effects of the immobilization conditions were studied, the thermal stability of the obtained immobilized CALB was evaluated. The immobilized CALB was then used to catalyze the glycerolysis reaction for DAG production. Reaction conditions were optimized and the reusability of the immobilized CALB was evaluated.

2. Materials and methods

2.1. Materials and reagents

Refined, bleached and deodorized corn oil was purchased from a local supermarket. SBA-15 with three different pore diameters (6.6, 8.1 and 12.5 nm) were purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). Novozym 435 (acrylic resin immobilized CALB) and CALB was kindly provided by Novozymes (Beijing, China). Glycerol with a purity of >99.0% was from Sinopharm Chemical Reagent Co., Ltd (Shanghai China). Tributyrin (>97%) for activity analysis and the standards of 1-monoolein, 1,3-diolein and triolein (>99.0%) for HPLC analysis were from Sigma (St. Louis, MO, USA). All other solvents and reagents were of analytical or chromatographic grade.

2.2. Immobilization of CALB onto SBA-15

The commercial extract CALB supplied by Novozymes (Beijing, China) had a protein concentration of 5.086 μg/mg, determined by the Bradford assay (Arica, Soydogan, & Bayramoglu, 2010). Required amounts of this extract were dissolved in 25 mM phosphate buffer, up to a total volume of 40 ml. Samples were withdrawn at this stage for the initial lipase activity (E_0) measurement. Then 100 mg of SBA-15 were added into the solution, and magnetically stirred at room temperature. After that, the suspensions were filtered and washed with the phosphate buffer. Supernatants were tested for the final lipase activity (E_f). Unless otherwise stated, the immobilized CALB samples were dried in a vacuum oven (presure at −0.093 MPa) at 25 °C for 2 h before activity analysis. Water content of the obtained immobilized CALB was at 17.59%. Samples with water content at 9.23% and 3.22% were obtained through being dried in a vacuum oven (presure at −0.093 MPa) at 25 °C for 4 h and 30 °C for 6 h respectively. Water content was determined by heating samples at 105 °C for 1 h, and the mass of water was the weight loss during the heating process.

2.2.1. Immobilization efficiency measurement

To determine the immobilization efficiency, samples were withdrawn as mentioned above, and hydrolytic activities were measured. A blank assay was also conducted to evaluate a possible lipase deactivation under the immobilization conditions. For this purpose, a solution of CALB was placed in a reactor under the same conditions of immobilization, but without the SBA-15 addition. The immobilization efficiency (IE) was calculated through the following equation (Liu, Fu, et al., 2012; Liu, Wang, et al., 2012):

$$\text{IE}\ (\%) = (E_0V_0 - E_fV_f)/E_0V_0 \times 100$$

where E_0 is the initial lipase activity (U/ml), V_0 is the initial volume of enzyme solution (ml), E_f is the lipase activity in the filtrate (U/ml), and V_f is the filtrate volume (ml).

2.2.2. Assay of enzymatic activity

The activity of the free or immobilized CALB was determined according to Wu et al. (2012) with some modifications. The tributyrin mixture consisted of 1 ml of tributyrin and 50 ml of phosphate buffer (25 mM, pH 7.0) was vigorously stirred at 40 °C. Then 1 ml of the free CALB solution or 10 mg of the immobilized CALB was added, and the mixture was continuously titrated with 0.1 M NaOH solution for 15 min to maintain a constant pH. Blank experiments were performed through same procedures but without lipase addition. One unit (U) of lipase was defined as the amount of lipase required to release 1 μmol of titratable free butyric acid per minute under the described conditions. The activity of the commercial CALB solution was found to be 4340.4 U/ml. All the experiments were conducted in triplicate.

2.2.3. Thermal stability

Thermal stability of SBA-15(6.6)-CALB with different water content was examined by measuring the activity of samples taken at regular time intervals and comparing the results with those at the beginning of the experiment, the initial activity was defined as 100% (Yang et al., 2013). The immobilized CALB samples were stored at 70 °C without incubation in any medium for a period of time and then withdrawn for activity assay. For comparison, sample of SBA-15(6.6)-CALB with water content at 17.59% was also incubated in hexane and phosphate buffer (pH 7.0) solution at 70 °C and then withdrawn at time intervals for activity measurement. In addition, thermal stability of the CALB solution was also evaluated. In this case, the CALB solution was incubated in phosphate buffer (pH 7.0 and the CALB concentration at 30.5 μg/ml) at 70 °C for 0 h, 0.5 h, 1 h, 2 h, 3 h and 4 h respectively. After that, the activity of the CALB solution was determined under the same reaction conditions as described above.

2.3. Characterization

Small-angle powder XRD was carried on Bruker (D4) advance diffractometer, using Ni-filtered Cu Kα radiation at 40 kV and 40 mA in the 2θ range of 0.5–8°, at scan speed of 0.2°/min. The