IMPACT OF IMMOBILIZATION SUPPORTS FOR POLYESTERS SYNTHESIS ACTIVITY OF CANDIDA ANTARTICA LIPASE B[a]

Atul S. Bhangale,[2†] Santanu Kundu,[1] Wenchun Xie,[2] William E. Wallace,[1] Kathleen M. Flynn,[1] Kathryn L. Beers[1] and Richard A. Gross[2]*

[2]Polytechnic Institute of NYU, Brooklyn, NY 11201
[1] Polymers Division, National Institute of Standards and Technology, Gaithersburg, MD 20899

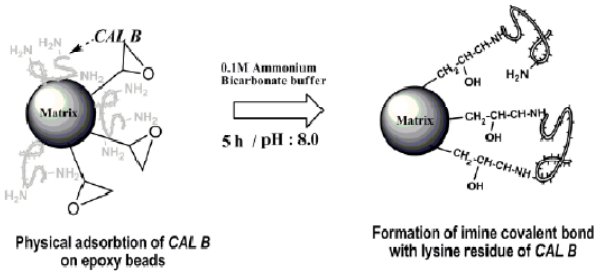
Introduction

Candida antarctica lipase B (CAL B) is a versatile biocatalyst with broad applications in industry and organic synthesis. Simplicity of use, low cost, commercial availability and recycling possibility makes CAL B an ideal tool for the synthesis and resolution of a wide range of compounds. Novozyme N435, a physically immobilized CAL B on Lewatit VP OC 1600, has been used to prepare high enantiopurity carboxylic acids, amines, alcohols and simple esters including polyesters. In the case of covalently immobilized CAL B, epoxy functionalized supports are known to be most suitable for industrial-scale immobilization of proteins.[1, 2] Epoxy supports have many advantages such as, better stability, and long-term storage ability. In addition, epoxy functionalized supports react with different functional groups (amine, thiol, hydroxyl groups) of proteins, yielding very stable protein-support bonding. A wide range of polymeric supports have been investigated in the past for lipase immobilization via different surface functional groups like amino, sulfhydryl, hydroxyl and epoxies. However, chemistry across these functional groups is known to affect the catalytic performance of the biocatalyst due to insufficient control over (i) multi-point covalent binding (ii) reduced active site accessibility or (iii) enzyme denaturation. Thus, it has often been difficult to achieve stable binding without compromising the enzyme activity. In order to achieve a balance between immobilized enzyme activity and minimal leaching, immobilization supports with different physico-chemical properties have been systematically studied. In the present study, we report immobilization of CAL B onto epoxy functionalized supports. CAL B was immobilized covalently on epoxy functionalized supports with different physico-chemical properties. Amongst them, Amberzyme and silanized silica supports were selected as potential candidates and were studied in detail. CAL B physically immobilized on Lewatit was used as a positive control to compare with the covalently immobilized CAL B systems. A series of measurements were conducted to evaluate the performance and stability of the covalently immobilized catalyst in polymerization of ε-caprolactone using Nuclear Magnetic Resonance (NMR) spectroscopy and Gel permeation Chromatography (GPC). Results show that increasing hydrophobicity of the matrix lead to increased enzyme loading. However, increasing the epoxy functional group density above 0.0013 moles/g of matrix, caused decrease in the activity of covalently immobilized CAL B.

In order to more systematically quantify the behavior of solid-supported catalysts a microreactor based approach was developed, wherein the activity and leaching of immobilized enzymes could be characterized in a continuous flow environment. As illustrated in Figure 2, an enzyme packed bed microchannel reactor was designed to better study the ε-caprolactone (ε-CL) ring opening polymerization (ROP) reaction. Microreactor-based tools are already well documented in literature as stand-alone devices for high-throughput biocatalyst screening and combinatorial materials science.[3,4] However, not many immobilized enzyme systems are studied in microchannel reactors so far. We used methods similar to those already developed to study other polymerizations in microchannels.[5,6] CAL B was used as a model enzyme to build a prototype reactor, since it is one of the more versatile and common enzymes with a wide range of applications. We believe that with the high versatility and superior control over reaction conditions, an enzyme packed bed reactor can provide a new platform to study a wide range of polymerization reactions via chemo-enzymatic routes.

Experimental*

Determination of CAL B purity: Determining the percent purity of the enzyme provides a rational basis for estimating the exact CAL B loading efficiency in the case of CAL B from different sources. Engineered CAL B, SP525, was obtained from Novozyme. The enzyme was separated from the broth by centrifugation, Ultrafiltration (5 KDa membrane), which also removes low molar mass impurities. The percent purity of the purified enzyme was determined by Experion automated electrophoresis system. The estimated purity of the purified enzyme was about 95%.

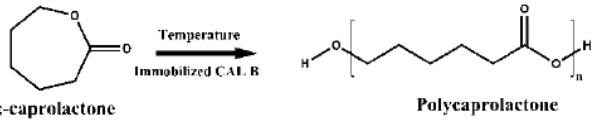


Scheme 1. Immobilization of CAL B on epoxy functionalized macroporous supports.

Immobilization of CAL B: The immobilization protocol used was as described by Chen et al (2007) with minor modifications and improvisations [7]. Functionalized epoxy supports were dried overnight at 37 °C in vacuum oven. Predetermined quantity of these supports were activated with ethanol for about 5 min and degassed to remove any entrapped air in the pores. 1mg/ml solution of previously purified CAL B, was prepared in 0.1 moles/L, pH 8.0 phosphate buffer and transferred to the pre-activated supports to maintain a 1:10 enzyme to support ratio. The mixture was placed in a shaking incubator at 15 radians sec^{-1} for pre-optimized immobilization period of about 5.0 h, at room temperature. After 5 h, the lipase-immobilized support was recovered by filtration and washed thrice with 0.1M phosphate buffer. The amount of lipase protein in the supernatant was determined by Bicinchoninic acid assay (BCA pierce kit). Serial dilutions of CAL B were run in parallel to obtain a standard curve for calibration. The amount of lipase adsorbed onto the polymeric supports was calculated as

$$q = (C_i - C_f)\, V/W \quad (mg/g)$$

Where, q is the total binding quantity (mg g−1), C_i and C_f are the concentrations of the initial soluble enzyme and final in the supernatant after immobilization and wash, respectively (mg/mL), V is the reaction volume (mL), W is the weight of the epoxy functionalized supports (g).



Scheme 2. Enzyme-Catalyzed Ring-Opening Polymerization of ε-caprolactone in Batch mode:

Immobilized CAL B preparations were dried thoroughly in the reaction vessel overnight at about 37 °C under vacuum (30 Torr). The weight ratio of CALB to ε-CL was maintained at 1:100 for all ring-opening polymerization experiments. Toluene-d_8 to monomer ratio was maintained 2:1(w/w). Toluene-d_8 was added to a 2-necked reaction vessel containing dried immobilized CAL B preparations under nitrogen. The reaction mixture was allowed to reach 70 °C before addition of ε-CL. Using a syringe, reaction aliquots were taken under constant nitrogen flush and immediately analyzed for percent monomer conversion. The aliquots were filtered through a 0.45 μm filter to remove any immobilized enzyme beads. Aliquots were analyzed by ^{1}H NMR at room temperature.

Measurements. Nuclear Magnetic Resonance spectroscopy (NMR). ^{1}H NMR measurements were performed on a Bruker DPX 300 spectrometer at 300 MHz. Chemical shifts was referenced to tetramethylsilane (TMS) at 0.00 ppm. NMR experiments were performed in deuterated solvents like d_8-toluene or $CDCL_3$ at 10 mg/mL for a total of 64 scans. NMR Data was collected and analyzed by MestRec-C.

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Gel Permeation Chromatography (GPC) analysis: Analysis of number average molar mass was performed at room temperature by GPC using SHIMADZU LC-20AD pump, Waters 717 plus auto sampler, Waters 2414 refractive index detector, and a PLgel 5um MIXED-D (300x7.5) mm column (Polymer Labs Ltd.) . Chloroform was used as an eluent at a flow rate of 1.0mL/min. Sample concentrations of 2 mg/mL to 5 mg/mL and injection volumes of 20 µl to 30 µl were analyzed. Eleven narrow polystyrene standards with molar mass ranging from 900K to 580 (from Polymer Lab Ltd.) were used to calibrate the GPC system. System calibration data and relative molar mass calculations were acquired and processed by Waters Empower software (with GPC option).

Results and discussion

The rate of CAL B loading and the maximum enzyme loading capacity of these supports is shown in Figure 1.

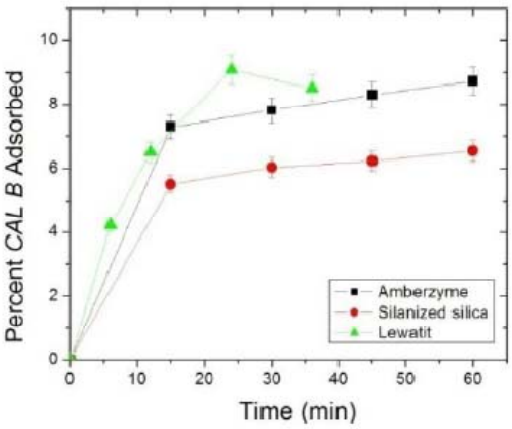

Figure 1. Rate of CAL B adsorption on different matrices. Each data point is an average of three readings. Error bars represent a standard deviation of less than 5%.

The activities of CAL B immobilized on these supports were evaluated for their performance in ε-caprolactone ring opening polymerization reaction. CAL B immobilized on Lewatit beads showed highest loading followed by Amberzyme and silanized silica particles (Table 1). As observed from Figure 1, the rates of CAL B adsorption on Lewatit and Amberzyme were almost identical; however, adsorption of CAL B on G192 was rather slow. Lewatit, Amberzyme and silanized silica had about 10%, 9% and 7% CALB loading respectively. The maximum enzyme loading for all these supports is tabulated in Table 1. CAL B immobilized on Lewatit beads and silanized silica beads showed almost similar monomer conversions at about 60 min.

Table 1. Performance of matrices used for CAL B immobilization assayed by ε- CL ROP. Each data point is an average of three readings.

Matrix	Functional group density (mM/gm)	Hydrophobicity	Enzyme loading (µgm) / mg matrix	Percent ε-caprolactone conversion (70°C, 60min)	M_n
Lewatit	N/A	+++	9.08	86.0 ± 4.0	8500 ± 500
Amberzyme	1.6	++	8.73	65.5 ± 3.0	5518± 100
G192	1.3	+	6.53	79.0 ± 4.0	5262 ± 200

CAL B physically immobilized on Lewatit showed a maximum ε-CL conversion of 86.0 % in 60 min at 70 °C in anhydrous toluene-d_8 , followed by G192 (76%) and Amberzyme oxirane (65%). However, even with higher conversion, the number average relative molecular mass (Mn) obtained for CAL B immobilized on G192 was significantly lower than Lewatit. Further, it was also observed that despite of lower enzyme loading capacity as compared to Amberzyme, G 192 had higher Kapp values than Amberzyme. However, Kapp values for covalently immobilized CAL B (Amberzyme & G192) were significantly lower than Lewatit.

Studies in enzyme packed bed microchannel reactor: A microreactor with 1 mm x 2 mm cross sectional area and a length of 260 mm was constructed in aluminum. Beads loaded with immobilized enzyme were packed inside the channel (Figure 2). ε-CL ROP reaction was used as a model reaction to evaluate the feasibility of studying polymerization reactions in packed bed microchannel mode. The microchannel was packed with CAL B immobilized on Lewatit VP OC 1600 (Novozyme N435) as shown in Figure 2. Reaction time was controlled by adjusting the flow rate of ε-CL and toluene over the packed bed, and the poly (ε-CL) solution was collected at the channel end for analysis of monomer conversion and product molar mass. To evaluate the performance of enzyme packed bed microchannel reactor, ε-CL ROP reactions were also conducted in traditional batch mode. Preliminary results suggest that, under predefined conditions of temperature and reaction time, a significant increase in the rate of reaction was observed with microchannel reactors as compared to batch reactor mode for the same stoichiometric ratio of enzyme: monomer : solvent.

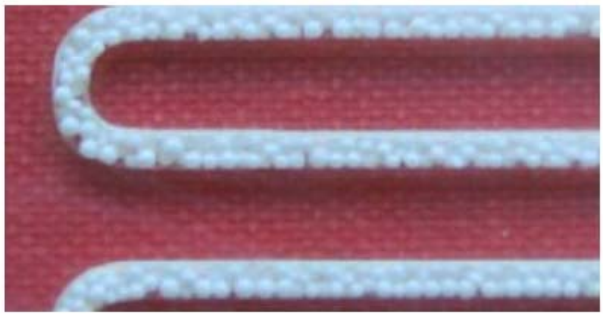

Figure 2. Immobilized enzyme beads packed in a microchannel reactor.

Conclusions

The effect of physico-chemical characteristics of three different matrices on CAL B polyester synthetic activity was studied. Although, covalently immobilized CAL B on silanized silica support (G192) and methacrylate (Amberzyme) showed lower performance as compared to physically immobilized CAL B on Lewatit, we believe that covalent immobilization of CAL B can prevent leaching of enzyme during the reaction. Designing strategies to minimize loss of enzyme activity on covalent immobilization can yield better and stable chemically bonded enzyme systems. This in turn can minimize product contamination from leached enzyme and increase the biocatalyst's reusability. Detailed study of leached enzyme quantification is required and will be addressed in our future communications. From our studies, enzyme packed bed microchannel reactors showed feasibility of this particular approach to screen novel biocatalyst and gain better control over experimental conditions. We also suggest that microreactor based approach can improvise the way traditional chemo-enzymatic synthesis are conducted.

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*Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.