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Ethanol pretreatment effect and particle diameter issues on the adsorption of *Candida rugosa* lipase onto polypropylene powder

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Abstract

Lipase from *Candida rugosa* (CR) was adsorbed onto low-molecular-weight polypropylene powder (PP). Fourier transform infrared (FTIR), scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX) techniques, and conversion yield of CR/PP-mediated-ethyl oleate synthesis in solvent-free media, demonstrated lipase adsorption onto PP and its affinity towards biggest polypropylene particles (590–1180 μm). An ethanol pretreatment was assayed in order to decrease the hydrophobic nature of PP. Long periods of ethanol pretreatment led to immobilised particles with different average size and associated activity. Molecular mechanics software packages were used to study the interaction between polypropylene and CR's lid.

Keywords: Ethanol pretreatment; Adsorption; Particle size; Interaction

1. Introduction

Enzyme immobilisation, particularly adsorption, is a practical and easy method that enables continuous use, reuse and separation of the enzyme from reaction products. In this study, *Candida rugosa* lipase was adsorbed onto low-molecular-weight PP previously treated with absolute ethanol (0.2% water).

Hyperactivation of lipase due to its adsorption onto hydrophobic supports of high specific surface area has been reported [1]. Lipases recognise these hydrophobic supports as solid interfaces and adsorb onto them in one of their open-activated conformations with their active site available [2,3]. Several conformational states exist between closed and totally open conformations. Using the protein data bank (PDB) data on the open (1crl) and closed (1trh) forms of *C. rugosa* and the PDB Viewer Program, possible intermediate states of the lid were modelled with software of the Chem3D 5.0 Ultra from Cambridge Soft [4]. Effects of a short model of PP on the steric energies of the conformations and presence of eight molecules of water in the near space of the lid were considered.

Several publications agree on the enhancement of PP's affinity towards lipase aqueous solution due to ethanol pretreatment of PP [5,6]. However, little attention has been paid on the severity of ethanol

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pretreatment and its influence on lipase content of immobilised biocatalyst. As it has been previously reported for *n*-octyl oleate direct esterification in solvent free medium, support characteristics like particle size greatly affect the initial rate of reaction and the extent of conversion [7]. At the author's knowledge, there is no report of the effect of PP's particle size on the adsorption of *C. rugosa* lipase. In this contribution, influence of particle diameter on adsorption efficiency and resultant catalyst activity is analysed. The effect of ethanol pretreatment on polypropylene particles size is also investigated.

2. Experimental

2.1. Materials

Low-molecular-weight polypropylene powder (30 000 g/mol) was obtained by metallocene-mediated-polymerisation. Lipase from *C. rugosa* AY (64 000 g/mol) was gently donated by Amano Enzyme Inc. (USA). Oleic acid was purchased from J.T. Baker, absolute ethanol was from Dorwil, and pH 7 buffer solution (di-sodium hydrogenophosphate) was from Merck.

2.2. Pretreatment of PP with ethanol-adsorption

Two different ethanol pretreatments were assayed on polypropylene. During ''Strong ethanol pretreatment'', particles with particle diameter (d_p) in the range of 210–1180 μm were subjected to 10 min at 800 rpm stirring followed by 50 min at 200 rpm stirring. ''Soft ethanol pretreatment'' involved particles with d_p between 590 and 1100 μm and only 3 min-stirring at 500 rpm. Later, 600 mg of lipase powder dissolved in 50 ml of pH 7 buffer were contacted with 1 g of ethanol-pretreated PP during 8 h.

2.3. Ethyl oleate synthesis

The CR/PP obtained catalysed solvent free ethyl oleate synthesis. Reaction was carried out in stirred 10 ml vials, at 45 °C. Three grams of oleic acid, 0.5 g of absolute ethanol (molar ratio ethanol/acid = 1) and 0.6 g of pH 7 buffer, were typically used. After 2 h of reaction samples were withdrawn and titrated with KOH.

2.4. Theoretical conformational study

Interfacial activation implies a *cis–trans* change of Proline 92 and changes in the conformation of several flap aminoacids. The complete CR lipase (534 aminoacids, 3 NAG) is very difficult to model. Interaction of a model of PP with the closed and open flap in absence of solvent was studied using molecular mechanics (MM2: Molecular Mechanics, Version 2) techniques and semiempirical methods (PM3: Parameterised model 3) in several steps of change (7–20 steps). Distribution of polar–non-polar side chain residues exposed to the solution is different in closed and open conformations of CR lipase's flap. With fixed initial and final conformations, eight possible steps of conformational change involving lid aminoacids groups were analysed. The step 1 and step 5 involve changes in hinge points. The calculations were performed (a) in vacuum, (b) in presence of a model of PP (24 atoms), (c) in presence of a model of PP + 8 water molecules contacted with polar side chains in the lid.

3. Results and discussion

Initial adsorption experiments included neither ethanol pretreatment of PP, nor initial particle diameter control. Produced biocatalyst was subjected to sieve analysis. FTIR and SEM–EDX analysis were performed on samples of CR/PP of highest and lowest average particle diameter. Fig. 1 illustrates IR spectra for equal amounts of two CR/PP fractions. Peaks found at 1118 cm^{-1} (C–O), between 1600 and 1750 cm^{-1} (C=O), and in 3423 and 3438 cm^{-1} (N–H), all attributed to *C. rugosa* lipase, verified lipase adsorption onto PP. However, spectra comparison showed that lipase does not adsorb onto particles of different size in the same proportion. Fraction 1 contained very small particles, while fraction 2 consisted of bigger CR/PP particles. Peaks due to N–H bonds and C–N bonds (between 1180 and 1360 cm^{-1}), and carbonyl group peak (between 1550 and 1650 cm^{-1}) are barely visible in fraction 1 spectra. C–O bond is also hardly noticeable in the

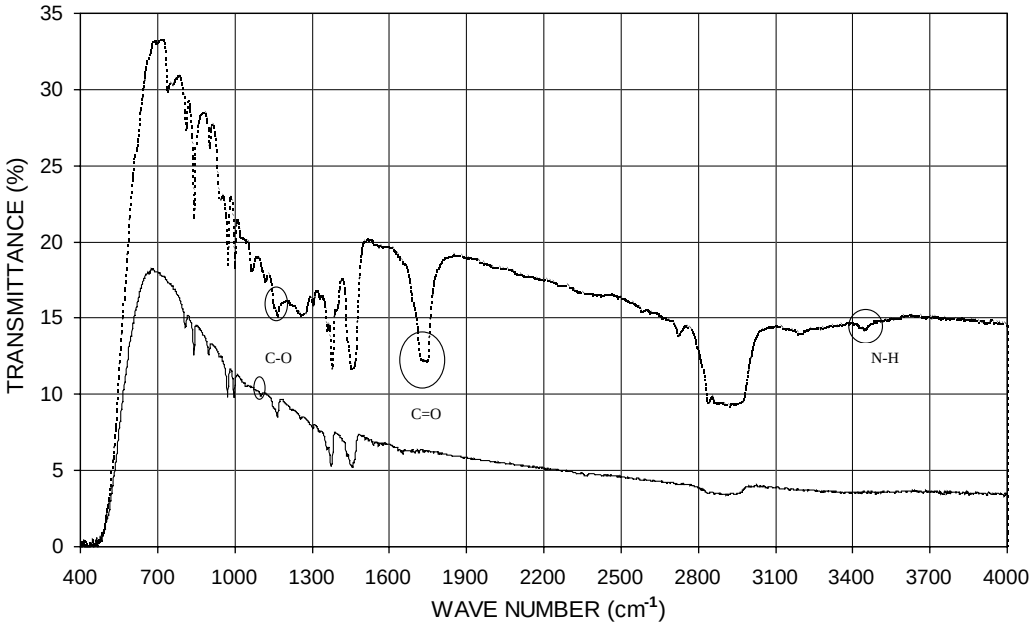

Fig. 1. FTIR spectrum of CR/PP. Full line corresponds to small particles and dotted line to bigger ones.

form of a little peak at 1128 cm^{-1}. Fraction 2 spectra (dotted line) exhibits the presence of higher amounts of adsorbed lipase. The small peak at 3452 cm^{-1} due to N–H bond, the noticeable C–O bond at around 1200 cm^{-1}, and the even more clearly seen carbonyl peak at 1743 cm^{-1}, they all demonstrate that *C. rugosa* lipase is present in larger amounts on bigger CR/PP particles. SEM–EDX techniques confirmed the effectiveness of the detailed adsorption method. EDX spectra of CR/PP showed an oxygen peak, only possible due enzyme presence (see Fig. 2a). Fig. 2b shows a SEM photograph of one of the biggest particles in 20 μm-scale.

Light dispersion due to colloidal PP particles, adsorption of buffer ions on PP and selective adsorption of lipase, cast serious doubts on the reliability of UV/Visible, FTIR and elemental nitrogen determination techniques for rigorous quantification of lipase content on the biocatalyst. However, the yield of ethyl oleate synthesis in the conditions detailed in Section 2.3, has demonstrated to be a very good indicative of lipase content. When 25 mg of CR/PP were used in the mentioned synthesis, particles with diameter lower than 210 μm led to one half of the yield obtained with CR/PP with d_p higher than 590 μm (2.1% versus 4.2%—the use of a very low amount catalyst accounts for the low values achieved). These observations (in addition to highest immobilisation efficiency and biocatalyst recovery percentage), led us to limit to 590 μm the minimum initial d_p of pretreated PP particles.

3.1. Severity of ethanol pretreatment and particle diameter

When PP was treated with ethanol prior to lipase immobilisation, time and rate of stirring were of crucial importance. ‘‘Strong ethanol pretreatment’’ led to great reduction of PP particles size, dramatically enhanced in subsequent eight-hour immobilisation process. Excessive colloid formation made it extremely difficult to get efficiency information by UV measurements, since several filtering stages led to buffer ions adsorption on CR/PP biocatalyst. When the obtained biocatalyst was tested in reaction, conversion yield was less than a third of that obtained with ‘‘soft ethanol pretreated PP’’ (2.8% versus 10.5%). Evidently, in agreement with FTIR analysis, colloidal particles had almost no adsorbed lipase leading to a notorious decrease in conversion yield.

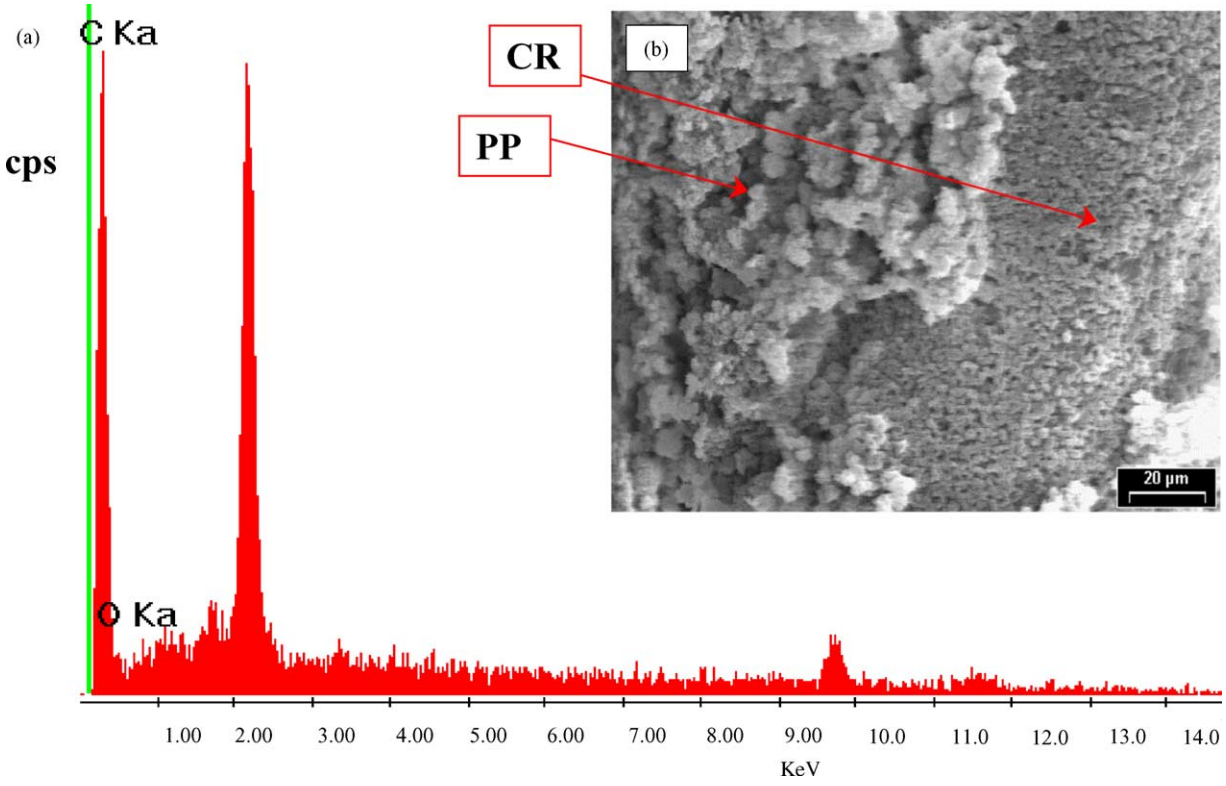

Fig. 2. (a) EDX of CR/PP. Oxygen presence, (b) SEM image of a big particle of CR/PP.

CR/PP obtained with soft ethanol pretreatment was much more uniform in size, and higher adsorption efficiency was achieved leading to an undoubtedly more active catalyst. In this case, UV measurements revealed that 20–30 mg of lipase were present in 100 mg of CR/PP.

3.2. Conformational steric energies

The steps analysed were—step 0: closed lid; step 1: Pro92 tran-Changes in Ser 91 up to Ala 89; step 2: change in Glu 88; step 3: change in Phe 87; step 4: changes up to Gln 83; ϕ, y, ψ conformational angles almost without change for Met 82, Val 81, Leu 80, Asp 79, Leu 78, Ala 77 and Ala 76; step 5: changes in Pro 65 and Gly 67; step 6: changes up to Tyr 69; step 7: open lid.

Table 1 shows the steric energies found in cases (a) and (b). In the closed lid, Lys 75 and Asp 79 side chains are exposed to solvent, and Leu 73 and Leu 78 turn to the inside of the protein core. This location of the side chain agrees with the known Hydrophobic effect (non-polar residues locate on the side chain inside the protein and polar side chain residues expose to solvent (water)), experimentally

Table 1
Steric energies (kcal/mol) found by minimisation for steps 1–7

Step	Vacuum	With PP model
0	−187.3	−103.1
1	−158	
2	−166.7	−84.7
3	−156.5	
4	−158.6	−94.3
5	−154.3	
6	−146.4	−91.8
7	−202.4	−128.8 parallel, −126.3 perpendicular

Energy differences between steps have physical meaning. Conformations are intermediaries not transition states.

found for proteins in water. In the case of the open lid, Leu 73, Ala 76 are exposed to solvent, while Lys 75, Asp 79 and Pro 74 turn to the inside of the protein core. This location of the side chains is experimentally found when an interface oil/water is present.

4. Conclusion

The influence of support average particle size on the adsorption of *C. rugosa* lipase onto polypropylene powder has been discussed. FTIR and yield of catalysed ethyl oleate synthesis showed that bigger particles of CR/PP contained more lipase than smaller ones. Ethanol pretreatment of the support led to a more uniform catalyst, higher adsorption efficiency, and increased solid recovery. However, long-time stirring periods led to very small particles with little adsorbed lipase. Not only PP size at the beginning of the immobilisation procedure, but also final CR/PP size deserves great attention. Additional reduction of CR/PP particles size during reaction complicates its recovery and prevents its re-use.

According to molecular mechanics calculation results, it seems that PP stabilises non-polar side chain exposure and polar side chain redirection. In addition, water presence reinforces the PP effect on the lipase lid (data not shown).

Acknowledgements

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