STUDIES ON OPTIMIZATION OF ENZYME CATALYZED PROCESSES

by

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DEPARTMENT OF CHEMISTRY

Submitted in fulfillment of the requirements for the degree of
Doctor of Philosophy

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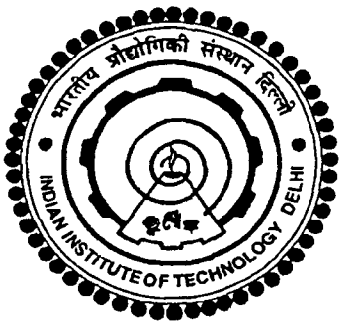

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Enzymes ② Biocatalysis

To
My Papa

CERTIFICATE

This is to certify that the thesis entitled, "**Studies on optimization of enzyme catalyzed processes**", being submitted by **Ms. Shweta Shah**, to the Indian Institute of Technology Delhi for the award of the degree of Doctor of Philosophy in Chemistry, is a record of bonafide research work carried out by her. Ms. Shweta Shah has worked under my guidance and supervision, and has fulfilled the requirements for the submission of this thesis, which to my knowledge has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full, to any other university or institute for award of any degree or diploma.

Date: 20|8|07

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Abstract

Enzymes are biocatalysts that can accelerate the rate of reaction while maintaining very high selectivity. Most of the synthetic reactions are performed in organic solvents. The realization that enzymes can also work in nearly anhydrous media has increased the prospects of employing enzymes in synthetic organic chemistry. Although enzymes can work in non-aqueous medium, they exhibit low reaction rates in non-aqueous media, almost 4-5 orders of magnitude lower than the aqueous media. This thesis focuses on the ways to obtain enzyme preparations that are highly active and selective in non-aqueous media.

Chapter 1 reviews the literature on different aspects of non-aqueous biocatalysis and various techniques involved in tailoring of enzymes for efficient performance in non-aqueous medium. Special emphasis has been given to the enzyme catalyzed transesterification of oils in organic medium for biodiesel production and use of ionic liquids as a reaction media for biocatalysis. Effect of ultrasound on enzyme performance in aqueous and non-aqueous media has also been reviewed. This chapter also provides a brief overview of nanobiocatalysts.

Chapter 2 describe the ways of preparation of cross-linked protein aggregates (CLEAs). It has been shown that by adding bovine serum albumin (BSA) as a proteic feeder facilitates the formation of CLEAs in the cases where the protein concentration in the enzyme preparation is low and/or the enzyme activity is vulnerable to glutaraldehyde cross-linking required to obtain aggregates. CLEAs of *Burkholderia cepacia* lipase (BCL) and penicillin G acylase (PGA) were prepared using this approach. CLEAs show

high activity in organic solvents as compared to the free enzyme powders. Lipase CLEA showed twelve-fold increase in activity over free enzyme powder when the CLEA was used in transesterification of tributyrin in isooctane. In the case of PGA, 86 % activity could be retained by using BSA as proteic feeder. CLEAs prepared without BSA lost 20 % activity after 8h at 45 °C whereas CLEAs with BSA retained complete activity. CLEAs prepared with BSA showed V_{max}/K_m of 6.3 min $^{-1}$ whereas CLEAs prepared without BSA had V_{max}/K_m of 17.4 min $^{-1}$ only. SEM analysis showed that CLEAs prepared in the presence of BSA were less amorphous.

In chapter 3 cross-linked protein coated microcrystals (CLPCMCs) were prepared which also showed enhanced performance (in terms of reaction rates) and higher thermal stability in organic media of different polarity. CLPCMCs of subtilisin *Carlsberg* (SC) and lipases from *Candida rugosa* (CRL) and BCL were prepared by cross-linking protein coated microcrystals (PCMCs). Their performances were compared with PCMCs which itself are considered high activity biocatalysts. These showed enhanced reaction rates for transesterification in organic solvents of different polarity as compared to PCMCs. CLPCMCs of SC were catalytically active till 80 °C in octane and gave 98 % conversion in 6 h while PCMCs gave only 58 % conversion in 8 h and conversions did not further increase with time. The fourier transform infrared (FTIR) spectra showed that alpha-helical content of CLPCMCs (unlike that of PCMCs) of SC were similar to what has been reported for free SC in water.

Chapter 4 describes the use of different biocatalysts for production of biodiesel. The monoethyl esters of the long chain fatty acids (biodiesel) were prepared by alcoholysis of *Jatropha* oil, a non-edible oil, by a lipase. The process optimization consisted of (a)

screening of various commercial lipase preparations, (b) pH tuning, (c) immobilization, (d) varying water content in the reaction media, (e) varying amount of enzyme used, (f) varying temperature of the reaction and (g) using high activity biocatalysts. The BCL lipase was found to give best conversion among the different lipases screened. The BCL lipase immobilized on Celite gave 98 % conversion at 50 °C in the presence of 3.5-5 % (w/w of the enzyme) water in 8 h. It was found that conversions were not affected if analytical grade alcohol was replaced by commercial grade alcohol. This biocatalyst could be used at least upto four times without loss of any activity. High activity biocatalysts were also evaluated for their catalytic efficiency for the preparation of biodiesel. CLEAs of BCL were used for transesterification of *Jatropha* oil. For the transesterification of *Jatropha* oil, while free enzyme powder required 8h and 50 mg lipase for obtaining 77 % conversion, CLEA required only 6h and 6.25 mg lipase for obtaining 98 % conversion. PCMCs of BCL were prepared by simultaneous precipitation of mixture of aqueous lipase solution and salts such as potassium sulphate by organic solvents. The enzyme coated micro-crystals so obtained, showed enhanced transesterification rates. Also, the PCMCs were stable at 60 °C whereas the free enzyme lost all its activity. The enzyme coated micro-crystals prepared from 50 mg of BCL gave 96 % conversion in just 90 min whereas free enzyme gave only 8 % conversion. Even PCMCs prepared from 3.12 mg of lipase gave 96 % conversion in 10 h at 60 °C whereas free lipase was inactive at 60 °C. The structure of micro-crystals was studied by AFM. The AFM picture confirmed the enzyme coating over the potassium sulphate crystals. These PCMCs are in the size range of 0.5–10 μm. These high activity biocatalysts were also evaluated for biodiesel preparation from high free fatty acid containing *Madhuca*

indica seed oil. In this case both the preparation gave high conversion. BCL powder gave 45 % conversion in 8 h using 50 mg lipase, CLEAs gave 92 % conversion in 2.5 h (using equivalent amount of 50 mg enzyme) and PCMCs gave 99 % conversion in 2.5 h while using same amount of enzyme.

In chapter 5 ionic liquids have been used as a useful reaction media for biocatalysis. This chapter describes the way the enzymes are dried before being used in ionic liquids and the performances of high activity biocatalysts (known to give high reaction rates in organic solvents) in these media. It is known that subtilisin shows poor transesterification activity in ionic liquids (ILs). This chapter, taking subtilisin as the system, describes approaches for biocatalyst preparations, which are capable of yielding higher/adequate transesterification activity in these media. Of all the approaches tried, enzyme precipitated and rinsed with *n*-propanol (EPRP) gave the best results (about 10,000 times increase in initial rates in 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]) over what was obtained with pH tuned lyophilized powders). In case of water soluble ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]), pH tuned lyophilized subtilisin did not show any transesterification activity. EPRP, however, gave an initial rate (of transesterification) of 2.78 mmoles mg^{-1} h^{-1}. Further, lipases from two different sources *Candida rugosa* (CRL) and *Burkholderia cepacia* (BCL) and protease α-chymotrypsin were formulated as enzyme precipitated and rinsed with organic solvents, organic solvent rinsed enzyme preparation, cross-linked enzyme aggregates (CLEAs) and protein coated micro-crystals (PCMC). These various lipase formulates were evaluated for the kinetic resolution of (±)-1-phenylethanol in ionic liquid [Bmim][PF_6] by transesterification with vinyl acetate. Of all the enzyme forms evaluated

(in the case of CRL), EPRP and PCMC showed the best results with 26 % (E value =153) and 53 % (E value =79) conversions respectively at 35 °C in 24 h. Carrying out this conversion with PCMC at lower temperature of 25 °C further improved the E value to 453 (with 44 % conversion in 12 h). For BCL, the acetone rinsed enzyme preparation (AREP), CLEA and PCMC performed equally well with % conversion of 50 and 99 ee_p (%) (E value > 1000) in just 2 h, whereas, the free lipase gave 8 % conversion. The pH tuned α-chymotrypsin has been found to be inactive in anhydrous ionic liquids [Bmim][PF_6] while PCMC, EPRP and PREP gave appreciable conversions. PCMC gave highest reaction rate and 51 % conversion in 18 h.

In chapter 6 multi-walled carbon nanotubes has been explored as immobilization matrix for lipases and also used for simultaneous refolding, purification and immobilization of xylanase. Retention of biologically active proteins on nanosized surfaces is a key operation in the context of bionanofabrication. Carbon nanotubes, in view of their high surface area, useful electronic, thermal and mechanical properties, constitute an important building block for fabricating novel functional biomaterials. Lipase from *Candida rugosa* (CRL) was found to be adsorbed on the multi-walled carbon nanotubes with very high retention (97 %) of biological activity. The immobilized biocatalyst showed 2.2 times and 14 times increase in initial rates of transesterification activity in nearly anhydrous hexane and water immiscible ionic liquid [Bmim][PF_6] as compared to lyophilized powder of enzyme. Presumably the interaction with hydrophobic surface of the nanotubes resulted in the conformational changes leading to 'open lid' structure of CRL. The immobilized enzyme was found to give 64 % conversion as opposed to merely 14 % conversion in 24 h for free enzyme for the formation of butylbutyrate by transesterification in nearly

anhydrous hexane. Similarly, in the ionic liquid [Bmim][PF_6], the immobilized enzyme gave 71 % conversion as compared to 16 % conversion by free CRL. The immobilized lipase also showed high enantioselectivity as evaluated by kinetic resolution of (±) 1-phenylethanol in [Bmim][PF_6]. While free CRL gave only 5 % conversion after 36 h, immobilized enzyme resulted in 37 % conversion with > 99 % enantiomeric excess. TEM and AFM studies of the immobilized biocatalyst showed that the enzyme is attached to the multiwalled nanotubes. Multi-walled carbon nanotubes were used as refolding aid for xylanase unfolded with 8 M urea. The hydrophobic surface of the nanotubes enabled the binding, refolding, purification and simultaneous immobilization of the enzyme. While 55 % activity could be regained while working with the denatured form of a purified preparation of xylanase, 92 % activity could be obtained with the commercial preparation of xylanase in 8 M urea. These activities were obtained with refolded xylanase bound to the carbon nanotubes. Hence an immobilization efficiency of 0.92 was observed. The FT-IR spectroscopy showed that α-helical content of xylanase decreased from 17 % to 14 %, β-sheet content increased from 53 % to 61 % and β-turns decreased from 20 % to 15 % upon immobilization on the nanotubes. The refolded xylanase molecule bound to the carbon nanotubes gave various secondary structure contents very similar to the native xylanase. .

Chapter 7 describes the effect of ultrasonic pre-irradiation of lipases (from *Burkholderia cepacia* and *Pseudomonas fluorescens*) on their activities in both aqueous buffer and organic solvents. It was found that both hydrolytic (esterase) and transesterification activities were enhanced. As practical application, it was found that % formation of butyl butyrate increased from 66 % to 82 % when pre-irradiated enzyme was used. Similarly,

79 % conversion of *Jatropha* oil to biodiesel with pre-irradiated enzyme was observed whereas untreated enzyme gave only 34 % conversion.

Circular dichorism (CD) spectra showed that while secondary structure of the enzyme remained unaffected during ultrasonication, the microenvironments of aromatic amino acids did change and there was some perturbation in the tertiary structure. SEM analysis showed significant morphological changes in the enzyme preparation as a result of ultrasonication.

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