BIOCATALYSIS BY HYDROLASES IN LOW WATER MEDIA

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by

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Dedicated to

Baba and Ma

who have provided unconditional support at every step of my life and made a lot of sacrifices so that I could pursue my goals in life

And

Prof. M.N. Gupta

who mentored me so that I could realize my potential, who believed and constantly made me believe that I can do better. I dedicate this to him as a token of appreciation for his patience and efforts in grooming me into a better researcher

CERTIFICATE

This is to certify that the thesis entitled **"Biocatalysis by hydrolases in low water media"** being submitted by **Mrs. Joyeeta Mukherjee** to the Indian Institute of Technology Delhi for the award of the degree of Doctor of Philosophy in Chemistry is a record of bonafide researched work carried out by her. **Mrs. Mukherjee** has worked under our supervision, and has fulfilled the requirements for the submission of the thesis which, to our knowledge, has reached requisite standard.

The results contained in this dissertation have not been submitted in part or in full to any other University or Institute for the award of any degree or diploma.

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ABSTRACT

The present thesis describes some efforts to improve the catalytic performance of the enzymes in general and hydrolases in particular in organic solvents. Even among hydrolases, lipases and proteases have been used more often in organic solvents and are also the biocatalysts chosen in these studies. The thesis is divided into seven chapters.

Chapter 1 provides the overview of the results reported so far on the use of enzymes in organic solvents.

Chapter 2 describes the molecular bioimprinting of hydrolases by precipitation and its functional consequences in low water media. In this work, precipitation with organic solvents was used to trap the lipases in 'open lid' form. The idea was to integrate a better "drying" approach with bioimprinting. Lipases from *Thermomyces lanuginosa* (TLL), *Candida rugosa* (CRL) and *Burkholderia cepacia* (BCL) were obtained in the 'open lid' form by adding surfactants. TLL imprinted with 0.05% cetyl trimethyl ammonium bromide (CTAB) showed 12-fold increase in the transesterification activity and was a better preparation to kinetically resolve (±)-1-phenylethanol. With CRL, bioimprinting with N-octyl-β-D-glucopyranoside (OG) gave 10-fold increase in the transesterification rates and resulted in reversal of enantioselectivity of CRL. Bioimprinted BCL was also a 7-fold better catalyst for transesterification as well as enantioselectivity. The bioimprinted TLL was also used to synthesize biodiesel from soybean oil and ethanol in solvent free medium. Enzyme precipitated and rinsed with organic solvents (EPROS) of TLL under optimized conditions gave 82% conversion to biodiesel in 4 h whereas the EPROS of TLL bioimprinted with CTAB and OG gave 99% biodiesel within 4 h. This chapter also describes the dual bioimprinting of enzymes by precipitation. A transesterification reaction involves an ester and an alcohol. Simultaneous imprinting with both the ester analog and the alcohol gave the best

biocatalyst for the transesterification reaction with an initial rate of 207 nmol min^{-1} mg^{-1}. These imprinted subtilisin precipitates showed 65-fold higher transesterification rates as compared to the rates obtained with the lyophilized powders. Dual bioimprinting was also tried in case of the lipase TLL by using the surfactant OG to open the lipase lid and by using the substrate ethanol to precipitate the lipase in the open lid form. By using very low amount of the enzyme (0.7 U), 98% conversion could be obtained in 48 h.

Chapter 3 describes the enzyme coated clusters of Fe_3O_4 nanoparticles for biocatalysis in low water media. Alpha chymotrypsin was precipitated over a stirred population of Fe_3O_4 nanoparticles in *n*-propanol. This led to the formation of enzyme coated clusters of nanoparticles (ECCNs). These clusters were also magnetic and their hydrodynamic diameter ranged from 1.2-2.6 μ. Optimized design of ECCN showed the highest initial rate of 465 nmol min^{-1} mg^{-1}protein which was about 9 times higher as compared to the simple precipitates with an initial rate of 52 nmol min^{-1} mg^{-1} protein. TLL was also precipitated over the nanoparticles to obtain lipase coated clusters of the nanoparticles. These ECCNs were used in the synthesis of biodiesel from soybean oil and ethanol in solvent free medium. Under optimized conditions the ECCN of TLL resulted in 99% conversion to biodiesel within 3 h with 14 U enzyme. Even 0.43 U enzyme resulted in 98% conversion but here the time required was 40 h. However, when the lipases in the open lid form were precipitated onto the nanoclusters, the resulting biocatalyst yielded 92% biodiesel in 7 h using just 1.75 U of the lipase.

Chapter 4 describes some further applications of immobilized lipases in the preparation of biodiesel and biolubricants. TLL immobilized on polyethylene imine (PEI) coated Fe_3O_4 nanoparticles proved to be a very efficient biocatalyst in the synthesis of biodiesel from soybean oil under solvent free conditions and 88% conversion to biodiesel was obtained in 4 h. Unfunctionalized mesocellular foam of silica was used for

immobilization of TLL. Two different approaches of immobilization were tried which gave rise to [(PEI-Fe_3O_4)-TLL]-MCF and [MCF-(PEI-Fe_3O_4)]-TLL. The second preparation performed better in synthesis of both hexyl butyrate and biodiesel. Lipases immobilized on commercial supports, for example Novozym 435 and RMIM (immobilized form of *Rhizomucor miehei* lipase) were tried for their performance in the synthesis of biolubricants by the transesterification of castor oil with higher alcohols like 1-hexanol, 1-octanol and 1-dodecanol. The effects of varying alcohol chain length, adding *n*-hexane as the solvent, temperature, oil: alcohol molar ratio and synergy of RMIM and Novozym 435 on final conversion have been studied. Under the optimized conditions 95%, 90% and 80% conversions to biolubricants could be achieved in 48 h using 1-hexanol, 1-octanol and 1-dodecanol respectively.

Chapter 5 describes the green synthesis of nanocomposites consisting of silver and protease alpha chymotrypsin. Ag-chymotrypsin nanocomposites were prepared by a two-step process. Step one consists of ultrasonication of a solution of silver ions along with alpha chymotrypsin. This was followed by exposure to UV/Visible light for a limited period of time. Ultrasonication alone formed very negligible number of nanoparticles of <100 nm size whereas light alone produced enough number but the size of the particles was >100 nm. The effects of pH, ultrasonication time, intensity, energy of light radiation and time period of exposure to different light radiations were studied. Ag-chymotrypsin nanocomposites of sizes ranging from 13 nm to 72 nm were formed using the synergy of ultrasonication and exposure to short UV radiation. Results show that ultrasonication promoted nuclei formation, growth and reduction of polydispersity by Ostwald ripening.

Chapter 6 describes how urea treated subtilisin can act as a very efficient biocatalyst for biotransformations in organic solvents. Subtilisin lyophilized from its solution in aqueous buffer in the presence of 6 M urea showed upto 50-fold increase (as

compared to lyophilized subtilisin not subjected to urea treatment) in its initial rate of a transesterification reaction in anhydrous *n*-hexane. Urea treated subtilisin had five times shorter half life during heating at 100 °C in hexane. The change in conformation was also reflected in its 92-fold higher activity at 15 °C as compared to merely 28-fold higher activity at 45 °C. The comparative enantioselectivity of urea treated subtilisin during kinetic resolution of 1-phenylethanol was expectedly lower. Its enantioselectivity during kinetic resolution of a natural substrate *N*-acetyl-DL-phenylalanine ethyl ester in hexane was higher. Urea treated subtilisin also showed higher catalytic promiscuity during an aldol condensation.

Chapter 7 describes the enzyme catalysed transesterification reactions carried out in saturated salt solutions. About 86% conversion in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester to *N*-acetyl-L-phenylalanine propyl ester catalysed by subtilisin was possible in saturated sodium bromide solutions after optimization of various process parameters. Another protease, alpha chymotrypsin was able to give a 77% conversion to the propyl ester in saturated sodium bromide as the reaction medium. In the case of lipases, CAL B worked best out of the five lipases screened for transesterification in this kind of medium. Again about 57% conversion could be obtained within 60 min. Novozym-435 gave a conversion of 72% propyl ester. These results show considerable promise in carrying out synthesis with hydrolases in aqueous media instead of organic solvents.

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LIST OF ABBREVIATIONS

a_w	Water activity
AOT	Bis(2-ethylhexyl)sulfosuccinatesodium salt
Ag-NP	Silver nanoparticles
BCL	*Burkholderia cepacia* lipase
CRL	*Candida rugosa* lipase
CAL A	*Candida antarctica* lipase A
CAL B	*Candida antarctica* lipase B
° C	Degree celsius
CD	Circular Dichroism
CLEAs	Cross-linked enzyme aggregates
CLECs	Cross-linked enzyme crystals
CLPCMCs	Cross-linked protein coated microcystals
CMC	Critical micellar concentration
CPME	Cyclopentylmethyl ether
CTAB	Cetyl trimethyl ammonium bromide
DLS	Dynamic light scattering
DME	Dimethoxyethane
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulphoxide
ee	Enantiomeric excess
ECCN	Enzyme coated clusters of nanoparticles
EPA	Environmental protection agency
EPROS	Enzyme precipitated and rinsed with organic solvents

FL	Fluorescent light
GC	Gas Chromatography
h	Hour
HPLC	High performance liquid chromatography
LUV	Long ultraviolet
min	minutes
mM	Millimolar
MML	*Mucor miehei* lipase
MCF	Mesocellular foam
OG	Octyl glucoside
PAH	Polyaromatic hydrocarbons
PCMC	Protein coated microcrystals
PEI	Polyethyleneimine
pNPP	*p*-Nitrophenylpalmitate
sec	seconds
SC	Subtilisin Carlsberg
SUV	Short ultraviolet
THF	Tetrahydrofuran
TEM	Transmission electron microscopy
TLL	*Thermomyces lanuginosus* lipase
Tyr	Tyrosine
TPP	Three phase partitioning
VOC	Volatile organic compounds