Improving catalytic performance of hydrolases in organic solvents: Applications in synthesis and kinetic resolution of organic compounds

BY

Abir B. Majumder

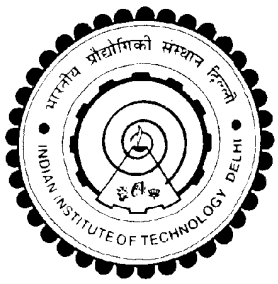
DEPARTMENT OF CHEMISTRY

Submitted in fulfillments of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

TO



INDIAN INSTITUTE OF TECHNOLOGY DELHI
NEW DELHI 110 016

To
My Parents

CERTIFICATE

This is to certify that the thesis entitled "Improving catalytic performance of hydrolases in organic solvents: Applications in synthesis and kinetic resolution of organic compounds", being submitted by Mr. Abir Baran Majumder, to the Indian Institute of Technology, Delhi for the award of the degree of Doctor in Philosophy in Chemistry, is a record of bonafide research work carried out by him. Mr. Majumder 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:

Dr. M. N. Gupta

Professor

Department of Chemistry

Indian Institute of Technology Delhi

New Delhi-110016

India.

ACKNOWLEDGEMENTS

I express my deepest gratitude to Prof. M. N. Gupta, my thesis supervisor, for his constant scientific guidance, ever encouraging word of wisdom and valuable suggestions towards successful completion of this work. I also thank him for all the facilities provided me to carry out the research work.

I am extremely grateful to Dr. N. G. Ramesh for his help and valuable discussions and for allowing me to avail his lab facilities through out my research work.

I sincerely thank Prof. A. K. Singh, Head of the Department for providing all the necessary facilities of the Department.

I sincerely thank Prof. Charusita Chackravarty, DRC chairperson, for all the help.

I am grateful to Prof. Tej P. Singh, Department of Biophysics, All India Institute of Medical Sciences (AIIMS) for helping me in X-Ray analysis.

I would like to thank Dr. S. N. Naik for helping me to get racemic alcohols.

I sincerely thank Dr. S. K. Khare for helping me by his lab facilities and also in non-academic matters.

Thanks from my bottom of heart go to Dr. Kalyani and Dr. Shweta, my seniors and coworkers who helped me to start working on the bench.

My sincere thanks to my seniors Dr. Aparna, Dr. Sulakshana, Dr. Smita and my colleagues Sohel, Pradeep, Kusum, Manali, Ghulam, Benu for their support at various stages during my research work.

I would like to thank Bhupinder, Debjit and Sushobhan for their assistance during my research work and Mr. Anjani Kumar, Ganga Sevak and Pyar Chand for their help.

My special thanks goes for Mr. Keshav (NMR, departmental), Mr. Munnalal (NMR, central) for helping me in taking NMR spectra and also to Aggarwalji and Sharmaji of Instrument lab for their help.

I would like to thank my friend Vivek for sharing our feelings during research.

I would like to thank my parents and my late grandfathers who always were inspiration for me to overcome difficult periods during my research work.

I would like to thank my wife for her moral support.

Last but never the least I would like to thank Council of Scientific and Industrial Research (CSIR) for providing me financial assistance during my research.

Abir Baran Majumder

ABSTRACT

The use of enzymes in organic synthesis has been attracting increasing attention over the last two decades. Most of such applications involve use of organic solvents as reaction medium. While enzymologists working in this area have carried out extensive work, the approach has not been still widely adopted by hard core organic chemists. The chemical industry has been also slow in switching over to the biocatalysis. One reason perhaps is the lyophilized powders or "straight from the vendor's preparations" which are favored by the organic chemists/ industry tend to give poor performance in terms of catalytic rates. Over the last decade, several approaches towards improving the catalytic performance of the enzymes in low water media have emerged. The present thesis describes the applications of such approaches in the context of synthesis and kinetic resolution of some useful organic compounds/ synthons. Hydrolases have dominated such applications. The present work has also been limited to the reactions catalyzed by hydrolases. The thesis is divided into nine chapters.

The first chapter is the introductory chapter which discusses the basic features of non-aqueous enzymology and strategies described in the literature on improving performance of enzymes in low-water media. The chapter also provides an overview of literature on kinetic resolution of racemates. The chapter essentially provides the background for the current work.

The second chapter deals with the synthesis of two important fragrances: the benzyl acetate and the citronellyl acetate. Benzyl acetate has been widely used as jasmine fragrance while citronellyl acetate apart from its characteristic fragrance is also known to be useful for its insect repelling properties. Benzyl acetate was prepared in a

solvent free medium. Use of vinyl acetate as acyl donor in transesterification of benzyl alcohol catalyzed by a commercially available lipase (Lipozyme RM IM) gave 100% conversion in 10 min. The excess acyl donor and the enzyme could be recovered and reused. Unlike the chemical catalytic processes, it produced no undesirable side product.

It has been shown earlier that removal of water molecules from enzyme by rinsing with *n*-propanol gives preparation (PREP) which shows higher activity in low water media. The present work evaluates PREP of the lipase (from *Rhizomucor miehei*) for kinetic resolution of (*R,S*)-*β*-citronellol. Generally primary alcohols with a remote chiral center are difficult to resolve. The acylating agent was vinyl acetate and the reaction was carried out in solvent free media. The PREP was indeed found to be more efficient and a 90 % enantiomeric excess of (*R*)-*β*-citronellyl acetate was obtained with 45 % conversion (enantioselectivity, $E = 42$). While the control with freeze-dried enzyme, could produce only an enantioselectivity of 13. FT-IR analysis of the amide I band showed that PREP had retained more α-helical content (28 %) as compared to the freeze-dried form which showed only 5 % α-helical content. Interestingly, the native lipase, by X-ray diffraction studies, is also known to have 28 % α-helical content. It showed that drying by propanol rinsing resulted in a better retention of native like structure which explained the higher activity in organic solvent.

In case of *Burkholderia cepacia* lipase, which was also tried to resolve citronellol, immobilization was carried out by cross-linking the protein by glutaraldehyde resulting in cross-linked enzyme aggregates (CLEAs). In addition to optimization of other known important parameters in such reactions, the enantioselectivity was controlled by changing the degree of cross-linking. An optimum cross-linking was

obtained with 10 mM glutaraldehyde which generated softer, less thermostable (half life 9 h at 55 °C in aqueous buffer), smaller (particle diameter < 5μm) but more enantioselective CLEAs. With this more improved immobilized formulation (over Lipozyme) a much better enantioselectivity (E =74) was obtained. Cross-linking with a higher concentration of glutaraldehyde (60 mM) resulted in a more rigid, more thermostable (half life 16 h at 55 °C, in aqueous buffer), bigger (particle diameter > 10μm) CLEAs which exhibited a poor enantioslectivity (E = 6). FT-IR study indicated the increase in β-sheet structure with the increase in cross-linking. This study indicates that, in CLEAs, optimum cross-linking brings in a decreased conformational flexibility which helps in improving the enantioselectivity.

While lipases are known to work very well with primary alcohols, the secondary alcohols with aliphatic straight chain (and their esters) are known to be poor substrates. Chapter 3 shows that the use of appropiate biocatalyst design improves catalytic performance of lipases towards such substrate. Two lipases were tried: a lipase from *Burkholderia cepacia* and from *Candida antarctica* (fraction B) of which the latter was found to give better results. Cross-linked protein coated microcrystals (CLPCMC) of *Candida antarctica* lipase B was found to bring about enantioselective transacetylation of racemic 2-pentanol, 2-heptanol and 2-octanol at 4 °C. In all the cases, the unreacted (*S*)-alcohol was obtained with > 99 % enantiomeric excess with an enantioselectivity (E) > 1000. When 2-pentanol was the substrate, ~50 % conversion to (*R*)-2-pentylacetate, with an enantiomeric excess > 99 % was achieved within 10 min. For this reaction, CLPCMC exhibited an initial rate which was >60 times faster and had 10 times better enantioselectivity as compared to the freeze-dried form. For other two alcohols the same conversion was achieved within 35 min. The unreacted enantioenriched (*S*)-alcohol was successfully purified by column

chromatography and the yield in all the cases was > 90 %. It was found that CLPCMC worked best under anhydrous conditions and dramatic decrease in rates and *E* values were observed when even a small amount of water was added to the reaction medium.

Chapter 4 deals with the enzymatic synthesis of tertiary alkyl butyrates. *Candida rugosa* lipase is one of the few lipases which shows at least limited catalytic activity towards tertiary alcohols and their esters. The transesterification of tributyrin with tertiary butanol and tertiary amyl alcohol were investigated. It was shown that precipitation of lipase by tertiary alcohols in the presence of a buffer with optimum ionic strength enhanced the catalytic rates by ~7 times to 134 μM /mg/h and 148 μM /mg/h for tertiary butyl alcohol and tertiary amyl alcohol respectively as compared to the rates obtained with lyophilized powders. The relative molar ratio of *t*-alcohol and the acyl donor (tributyrin) was optimized. For tertiary butanol the ratio was 2:1 and for tertiary amyl alcohol it was found to be 4:1. Octane was found to be the best solvent for *t*-butyl butyrate synthesis, while for *t*-amyl amyl butyrate, addition of solvent was found to decrease the rate. Thus, while lyophilized powders gave the initial rates as 16.6 μM /mg/h and 20 μM /mg/h with the alcoholysis by tertiary butyl alcohol and tertiary amyl alcohol respectively, the combined optimization in terms of biocatalyst formulation and medium engineering enhanced corresponding initial rates to 671 μM / mg/h and 597 μM /mg/h.

Chapter 5 describes attempts at the stereoselective synthesis of (*R*)-1-phenylethyl –*β*-D-galactopyranoside. A *β*-galactosidase (from *Aspergillus oryzae*) preparation, EPRP (enzyme precipitated and rinsed with propanol), obtained by the removal of bulk water by precipitation with *n*-propanol, showed higher transgalactosylation activity

than the lyophilized formulation. While the lyophilized formulation exhibited an initial rate of 575 μmol/ mg/h with an *E* value of 36 for the synthesis of (*R*)-(1-phenylethyl)- *β*-D-galactopyranoside, the EPRP exhibited ~2 times higher initial rate with a better enantioselectivity (*E* = 80). The reaction was performed initially in a solvent containing 5 % DMF and 5 % water and 90 % acetonitrile. The presence of water (both added and generated during galactosylation) was found to affect the synthetic rate and the enantioselectivity. Substituting DMF by DMSO and addition of optimized amount of molecular sieves to absorb water generated during the reaction led to an enantioselectivity, *E* > 1000 for the stereoselective synthesis of (*R*)-(1-phenylethyl)-*β*-D-galactopyranoside, starting from racemic 1-phenylethanol. This strategy was successfully applied with racemic 2-octanol as well. Under similar conditions, (*R*)-(2-octyl)-*β*-D-galactopyranoside was formed with an enantioselectivity, *E* = 38. FT-IR study confirmed that EPRP had retained the α-helical content of the native structure better than the lyophilized form.

Chapter 6 deals with the kinetic resolution of racemic pregabalin. The racemic pregabalin (a pharmaceutically important gamma amino acid) was converted to its methyl ester by acid catalyzed esterification. Alcalase (an industrial formulation of subtilisin Carlsberg from *Bacillus licheniformis*) at 30ºC within 2.5 h converted racemic pregabalin methyl ester to (*R*)-pregabalin with 51 % conversion and > 98 % ee. The unreacted (*S*)-ester was chemically hydrolyzed to (*S*)-(+)-pregabalin (>99 %). Attempts were made to reverse the enantioselectivity of alcalase so that the enzymatic hydrolysis gives the desired (*S*)-pregabalin directly. For this, as a first step, protease molecule was made more flexible by three phase partitioning, incubated with (*S*)-pregabalin at 70 °C for sufficient time to imprint and form an "(*S*)-preferring"

conformation, and then cross-linked with glutaraldehyde to "freeze" the conformation. This formulation in a reaction medium containing 40 % acetone in buffer at 5 °C did show an improved (*S*)-stereopreference and 51 % of enantiomeric excess of (*S*)-pregabalin was obtained at 50 % conversion within 16 h. Thus by this process an extremely high (*R*)-enantioselective (enantiomeric excess > 99 %) alcalase could be converted to a form which showed a reverse (*S*)-enantiopreference (enantiomeric excess ~50 %) towards pregabalin.

Candida rugosa lipase has broad substrate specificity and consequently is extensively used in the synthesis of esters or kinetic resolution of racemic alcohols. However, one problem with *Candida rugosa* lipase (and some other lipases) is that it gets easily deactivated in presence of vinyl esters which are unmatched in producing an excellent transacylation rate or a better enantioselectivity. The enzyme deactivation is due to the formation of acetaldehyde which is generated from the vinyl esters by enzymatic hydrolysis. In chapter 7, it is shown that a *Candida rugosa* lipase aggregate cross-linked in the presence of bovine serum albumin, did not show deactivation during transacetylation with vinyl acetate. A series of benzyl alcohols, viz. benzyl alcohol, 3,4-dimethoxybenzyl alcohol and racemic 1-phenylethanol were taken as the substrate. The formulation, under the same reaction conditions, exhibited 4-30 fold enhancement in the reaction rate depending upon the alcohol chosen as compared to the enzyme immobilized on celite or lyophilized preparation. Thus when benzyl alcohol was taken as the substrate, 82 % conversion to benzyl acetate could be achieved within 3h while both the native and the celite immobilized lipase could produce a conversion < 20 % during the same period of time. For, 3,4-dimethoxy benzyl alcohol the novel preparation exhibited an initial rate of 37,894 μM/ mg/ h

which was 95 times higher than the rate exhibited by the free enzyme (400 μM/ mg/ h). The racemic 1-phenylethanol, underwent a more efficient enantioselective transacetylation with this formulation than the enzyme immobilized on celite. Thus, with the enzyme immobilized on celite, after 24 h only 18 % conversion with an 83 % enantiomeric excess could be obtained while with the CLEA, 38 % conversion was obtained with a marginal loss of 3 % in enantiomeric excess value. Furthermore, the preparation could be reused up to five cycles with a loss of 40 % of its initial activity.

Enzymes from thermophiles have a greater ratio of arginine to lysine residues. This observation was used for enhancing the stability of serine proteases subtilisin and α-chymotrypsin. The CLEAs of three proteases viz. subtilisin, α-chymotrypsin, trypsin were prepared in the presence of an optimized amount of arginine. In all the cases the arginine grafted CLEAs showed a significant increase in thermal stability in aqueous solution and in water miscible organic solvents (60 % v/v in water). The arginine grafted CLEAs of subtilisin exhibited a half life of 72 h at 55 °C which was 36 times higher as compared to the value obtained with free enzyme. The half lives of the CLEAs of α-chymotrypsin and trypsin, at 55 °C, were found to be 9 h and 25 h while with the free enzymes the half life values were < 20 min. Under anhydrous condition arginine grafted CLEAs exhibited a significantly higher rate of transesterification as compared to the corresponding CLEAs. In presence of three water miscible organic solvents viz. acetonitrile, acetone and dioxan, arginine grafted CLEAs of subtilisin showed half lives ranging from 234-360 min while for the free enzyme, these values were within 30-40 min. The arginine grafted CLEAs appeared morphologically different from ordinary CLEAs under Scanning Electron Microscope. The arginine grafted CLEAs obtained from subtilisin were of smaller size (diameter 2-5 μm) and

more spherical in nature as compared to the CLEAs obtained from the enzymes α-chymotrypsin and trypsin.

The last chapter deals with a promiscuous reaction exhibited by *Candida antarctica* lipase B under anhydrous conditions. When Novozym 435 (an immobilized formulation of CAL B) was used for transacetylation of an enol of a tricyclic 1,3-diketone with vinyl acetate, it unexpectedly gave a condensation product. This promiscuous reaction was further studied. Thus 5-hydroxy-endo-tricyclo[5.2.1.0 ^{2,6}]deca-4,8-dien-3-one with acetaldehyde (enzymatically produced from vinyl acetate *in situ*) under low water conditions, in presence of 10% organic co-solvent (*N,N*-dimethylformamide or pyridine), gave a bis-adduct formed by an aldol type reaction. Even though the condensation reaction also occurred with pyridine (acting as a base catalyst) in presence of acetaldehyde and in the absence of enzyme, the reaction was very slow as compared to the enzymatic process. Thus, while the non enzymatic process took 4 days to achieve 100 % conversion; in presence of enzyme it was possible within 4 h. The enzyme also catalyzed similar reactions when acetaldehyde and propionaldehyde were directly used along with the tricyclic ketone.

TABLE OF CONTENTS