USE OF HIGHLY ACTIVE AND /OR REUSABLE BIOCATALYST DESIGNS FOR BIOTRANSFORMATIONS

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by

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CERTIFICATE

This is to certify that the thesis entitled "**Use of highly active and/or reusable biocatalyst designs for biotransformations**" being submitted by **Mrs. Benu Arora (nee Monga)** 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. She 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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Benu Arora (nee Monga)

Abstract

Use of enzymes as catalysts for synthetic reactions has expanded greatly in the last few decades. The phenomenon of enzyme promiscuity has further increased their application for white biotechnology. Of the various classes of enzymes, hydrolases (particularly lipases and proteases) are more often used for this purpose. This thesis explores the catalytic promiscuity of some high activity biocatalyst designs prepared from these enzymes for catalyzing organic reactions. This thesis is divided into six chapters.

Chapter 1, the introductory chapter reviews the relevant literature related to enzyme promiscuity and the organic reactions covered in this work.

Chapter 2 deals with enhancement of rates for hydrolase-catalyzed aldol reaction. Aldol reaction of *p*-nitrobenzaldehyde and acetone catalyzed by lipases has been reported to be a slow reaction; often extending over a period of 5-6 days to achieve > 90% conversion. In this chapter, a few simple approaches were used to speed up the same reaction. An initial optimization of ratio of water: acetone present in the reaction medium revealed that a 1: 1 mixture gave maximum conversion. At very low acetone concentrations, two side products: *p*-nitrobenzyl alcohol and *p*-nitrobenzoic acid were also formed (this reaction investigated in detail is described in the subsequent chapters). Effect addition of a few polar organic co-solvents to the reaction medium at varying concentrations was studied. DMSO at a concentration of 30% (v/v) w.r.t. the total reaction volume was found to be the best co-solvent. Screening of a number of commercially available lipases was then carried out. As a result of these optimizations, >90% conversion was attained after just 24 hours. The same reaction under the optimized conditions was then extended to the proteases. Finally, scope of this reaction was expanded by using various substituted benzaldehydes as substrates.

Chapter 3 describes the lipase catalyzed Cannizzaro-type reaction with substituted benzaldehydes in water. Novozyme 435 (immobilized *Candida antarctica* lipase B) was found to catalyze the disproportion of *p*-nitrobenzaldehyde to *p*-nitobenzyl alcohol and *p*-nitrobenzoic acid without addition of any external redox reagent. This reaction was found to occur in sodium phosphate buffer, pH 7.0 at 30 ° C. However, the reaction followed an unusual pattern of formation of products. In the beginning alcohol was formed as the major product (around 60% conversion was obtained after 6 hours) while the acid appeared only in traces. Thereafter there was a decline in the amount of alcohol and a simultaneous increase in acid formation, with the two products becoming almost equal after 24 hours. Thereafter, the lipase catalyzed oxidation of the alcohol was found to convert all the alcohol to the acid. Evaluation of the scope of this reaction was carried out by trying various substituted benzaldehydes as substrates. The addition of organic solvents such as DMSO, acetonitrile and CPME to the reaction of *p*-nitrobenzaldehyde catalyzed by Novozyme 435 resulted in almost complete inhibition of the acid formation.

Chapter 4 deals with the reduction reaction of substituted benzaldehydes catalyzed by Novozyme 435. As discussed above, adding organic solvents to the lipase catalyzed Cannizzaro-type reaction resulted in the exclusive formation of *p*-nitrobenzyl alcohol from *p*-nitrobenzaldehyde. To begin with, a wide range of organic solvents was screened to select the one that resulted in maximum conversion to the alcohol.

Cyclopentyl methyl ether was chosen for further work as this solvent gave maximum conversion to *p*-nitrobenzyl alcohol. CPME is well recognised as a green reaction solvent. Thus the lipase catalyzed reduction reaction was carried out under environmentally friendly conditions. A stepwise optimization of the substrate concentration, enzyme concentration and reaction temperature was then carried out. Under the optimized conditions, it was possible to convert about 80% of *p*-nitrobenzaldehyde to *p*-nitobenzyl alcohol. Finally using the same

optimised reaction conditions, a range of substituted benzaldehydes were reduced to their corresponding benzyl alcohols in high yields.

Chapter 5 describes the lipase catalyzed reactions of phenylglyoxal in aqueous media. Phenylglyoxal, an α-keto aldehyde is known to undergo intramolecular Cannizzaro reaction in the presence of chemical catalysts forming mandelic acid. In the present chapter the Novozyme 435 catalyzed reaction of phenylglyoxal was carried out in sodium phosphate buffer, pH 7.0. This reaction resulted in formation of mandelic acid along with phenylglyoxylic acid. The latter was formed as the major product due to the enzyme catalyzed oxidation of phenylglyoxal under the reaction conditions.The influence of polar organic co-solvents on this reaction was also evaluated. Addition of co-solvents such as DMF, DMSO, acetonitrile and dioxane to the reaction medium was carried out. Interestingly, adding the co-solvents increased the amount of mandelic acid formed during the reaction as compared to that formed in totally aqueous condition. However, the oxidation product i.e. phenylglyoxylic acid continued to be formed as the major product in all these cases as well.

Chapter 6 deals with condition promiscuity of lipases and its application to the synthesis of glucose esters. High activity biocatalyst designs of *Candida antarctica* lipase B were used to catalyze the transesterification reaction of D-glucose and vinyl acetate in low water media. Acetylation of D-glucose by vinyl acetate using CAL-B produces both 6-*O*- acetyl-D-glucose as well as 3, 6-*O*-diacetyl-D-glucose, with varying degrees of regioselectivity depending on the reaction conditions. Cross-linked enzyme aggregates (CLEAs), protein coated microcrystals (PCMCs) and cross-linked protein-coated microcrystals (CLPCMs) prepared from *Candida antarctica* lipase B were used in different solvents. In acetone as the medium, CLEA and CLPCMC gave better overall conversions than PCMC, but the reactions catalyzed by these were less regioselective. With CLPCMC and CLEA, initial rates of 366.1 and 353.5 μmol $min^{-1}g^{-1}$ were obtained respectively.

Next, mixed solvents were used as the reaction medium. Binary mixtures of *t*-amyl alcohol and DMSO (30% v/v) were initially chosen. The two biocatalyst designs: CLEA and CLPCMC which had performed better earlier were used here. Initial rates of glucose conversion increased to 3636.0 and 3333.0 μmol $min^{-1}g^{-1}$ using CLEA and CLPCMC respectively. A solvent premixing protocol was found to give still higher initial rates of glucose ester formation. Under these conditions initial rates of 7886.9 μmol $min^{-1}g^{-1}$ and 7447.5 μmol $min^{-1}g^{-1}$ were achieved using CLEA and CLPCMC. Overall, >90% conversion of glucose was attained within 1.5 hours of the reaction. Moreover, the monoacetate was formed regioselectively in these cases.

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Ala	Alanine
Arg	Arginine
CALA	*Candida antarctica* lipase A
CALB	*Candida antarctica* lipase B
° C	Degree celcius
CLEAs	Cross- linked enzyme aggregates
CLPCMCs	Cross-linked protein coated microcystals
CPME	Cyclopentylmethyl ether
DCM	Dichloromethane
DME	Dimethoxyethane
DMF	N, N-Dimethylformamide
DMSO	Dimethylsulphoxide
ee	Enantiomeric excess
EPRP	Enzyme precipitated and rinsed with propanol
GA	Glutaraldehyde
GC	Gas Chromatography
h	Hour
His	Histidine
HPLC	High performance liquid chromatography
Leu	Leucine
Lipase M	*Mucor javanicus* lipase
MCR	Multi-component reaction

mM	Millimolar
N435	Novozyme 435
NMR	Nuclear magnetic resonance
PCMC	Protein coated microcrystal
PPL	Porcine pancreatic lipase
pNPP	*p*-Nitrophenylpalmitate
Pro	Proline
Ser	Serine
THF	Tetrahydrofuran
TLL	*Thermomyces lanuginosus* lipase
Tyr	Tyrosine
Val	Valine