STUDIES ON PROMISCUOUS REACTIONS CATALYSED BY LIPASES

BY

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on promiscuous reactions catalysed by lipases**", being submitted by **Ms. Manali Kapoor**, 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 her. Ms. Kapoor 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.

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ABSTRACT

The use of enzymes in biotransformation has been attracting increasing attention over the last few decades. Recently, biological promiscuity of enzymes has attracted considerable attention. This discovery makes enzymes even more useful for biotechnological applications as well as in organic synthesis. Protein engineering and directed evolution have yielded valuable results in this area but from the practical point of view, the biological promiscuity of commercially available enzyme preparations is more attractive.

Chapter 1 is the introductory chapter and reviews relevant literature in the areas relevant to the thesis and thus essentially provides the background for the current work.

In Chapter 2, three phase partitioning (TPP) of lipases was carried out and treated lipases were then used to study a promiscuous reaction (aldol reaction between 4-nitrobenzaldehyde and acetone) catalysed by lipases. TPP is a strategy with a wide range of applications including protein purification and enhancement of catalytic activity of enzymes. TPP treatment of lipases from *Candida rugosa* and *Thermomyces lanuginosus* was carried out and the effect of variation of water concentration during the aldol reaction catalysed by untreated and TPP treated lipase was investigated. TPP treated *Candida rugosa* lipase at 30 % (v/v) water concentration gave 100 % conversion in 54 h whereas with untreated enzyme only 28 % conversion was obtained in the similar time period. At 40 % (v/v) water concentration, TPP treated *Candida rugosa* lipase gave about 100 % conversion in 48 h whereas untreated one gave 66 % conversion. With TPP treated *Thermomyces lanuginosus* lipase, 99 %

conversion was obtained in about 48 h at 30 % (v/v) water concentration whereas with untreated enzyme 45 % conversion was obtained at 30 % (v/v) water concentration.

Morita-Baylis-Hillman (MBH) reaction is of great synthetic utility as it yields a highly functionalized product with considerable atom economy. It has been reported by earlier workers that while bovine serum albumin could catalyse MBH reaction between 4-nitrobenzaldehyde **(I)** and 2-cyclohexen-1-one **(II)** to a limited extent (conversion up to 35 %), most of the lipases failed to catalyse this reaction to any significant extent. In chapter 3, it is reported that with change of the reaction medium from aqueous to aqueous-organic co-solvent mixtures, lipases can catalyse the MBH reaction. However, it was found that the aldol product is also formed simultaneously. Furthermore, it was found that the catalytic reaction could be carried out in an enantioselective manner under optimized conditions. Lipase from *Burkholderia cepacia* (BCL) was found to catalyse the reaction between **(I)** and **(II)**. Two products were obtained: the product of the MBH reaction and an aldol product. The total conversion varied with change in the ratio of the reactants and was highest (24 % after 24 h) when (I) and (II) were in the molar ratio of 1: 15. The different ratios of the MBH product and the aldol product were obtained when the ratio of the two reactant concentrations was varied. Different lipases were screened under the same conditions with (I): (II) in the molar ratio of 1:15 (the ratio of the reactants which gave maximum overall conversion). *Burkholderia cepacia* lipase turned out to be the best for obtaining maximum total conversion (24 % after 24 h). However, different lipases gave different ratios of MBH product to aldol product. *Mucor javanicus* lipase (MJL) gave almost equal percentage of two products; 9.7 % MBH product and 8.3 % aldol product. To examine the effect of DMSO concentration in the reaction medium, percentage of DMSO was varied over a wide range. At the end of 24 h, *Mucor*

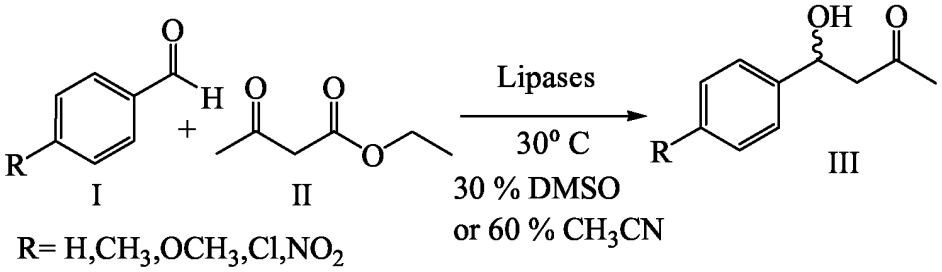
javanicus lipase gave even marginally higher amount of MBH product as compared to the aldol product (1.2 times) at 30 % (v/v) DMSO. The ratio of aldol product to MBH product was highest with BCL at 50 % (v/v) DMSO. After 72 h, BCL gave 96 % conversion with 50 % DMSO; 80 % of this was aldol product and MBH product accounted for 16 %. The highest amount of MBH product obtained was 33 % with MJL and with 30 % (v/v) DMSO.

Enantiomeric excess (*ee*) was also measured. Enantiomeric excess (*ee*) is defined by the following expression:

ee_S = ([1b]- [1a]) / ([1b]+ [1a]) where [1a], [1b] are the concentrations of enantiomers 1a and 1b. Similarly the product enantiomeric excess would be ee_P = ([2a]- [2b]) / ([2a]+ [2b]). The ee values are always expressed in %; so the ee values in fractions obtained by the above expressions are multiplied by 100.

The highest enantiomeric excess, *ee* (65 % of *d* form) for the MBH product was obtained with BCL at 30 % (v/v) DMSO. On the other hand, 79 % *ee* of the aldol product was obtained with MJL and again at 30 % (v/v) DMSO.

The decarboxylative aldol reaction is an important C-C bond formation reaction in organic synthesis. Chapter 4 investigates the decarboxylative aldol reaction between 4-nitrobenzaldehyde and ethyl acetoacetate (Scheme 1) catalysed by few lipases and a protease alcalase in aqueous-organic co-solvents mixtures.



Scheme 1

Lipase catalysed reaction between 4-nitrobenzaldehyde and ethyl acetoacetate has been earlier carried out in nearly anhydrous acetonitrile with *Candida antarctica* lipase B (CALB) to produce an aldol product. This was reported as a promiscuous reaction catalysed by a lipase. However, recent work showed it to be a normal reaction wherein CALB catalyses hydrolysis of ester resulting in the free acetoacetic acid, which then reacts with 4-nitrobenzaldehyde to give the aldol product/Knoevenagel product (presumably uncatalysed by the enzyme). In any case, in view of the synthetic utility of the reaction, it was thought worthwhile to further investigate this system. Retaining acetonitrile as a co-solvent, the identical decarboxylative aldol reaction was studied in the presence of much higher concentrations of water. Minimum concentration of 60% acetonitrile was required to get 100 mM 4-nitrobenzaldehyde concentration in reaction mixture. Organic co-solvents used at that concentration often denature enzymes. However, all the enzymes screened [*Mucor javanicus* lipase, CALB (free and immobilized), *Rhizomucor miehei* lipase (free and immobilized) and *Candida rugosa* lipase] were found to catalyse the decarboxylative aldol reaction in aqueous-60% acetonitrile co-solvent mixture. 98% conversion was obtained in 168 h with both free and immobilized forms of CALB. Various additives like triethylamine (NEt_3) and imidazole were added to decrease the time of conversion. When the reaction with CALB was carried out in the presence of increasing concentrations of imidazole, the initial rate of the decarboxylative aldol reaction was also found to increase. The maximum % conversion reached was between 97-99 % in all the cases. However, the time required to reach this maximum % conversion decreased as the imidazole concentration was increased. Other solvents (more polar than acetonitrile) DMSO, DMF and dioxane helped in obtaining 4-nitrobenzaldehyde at 100 mM concentration solution at 30 % v/v co-solvent

concentration. However, DMSO gave higher initial rates than the other two co-solvents. *Mucor javanicus* lipase showed the next best % conversion among enzymes (in the absence of any additive). Hence, this enzyme as well as CALB was tried in 30 % DMSO. Here again 10 mol % of NEt_3 as an additive increased the % conversion. In 30 % DMSO, both *Mucor javanicus* lipase and CALB gave identical conversions (54 %) in 90 h. The enantioselectivity of *Mucor javanicus* lipase was opposite to that of CALB and showed higher enantioselectivity (20 % *ee*) than CALB.

When a commercial protease preparation alcalase was used to catalyse this reaction, 99 % conversion was obtained in aqueous (35 % v/v)-DMSO co-solvent mixture whereas 98 % conversion was obtained in aqueous (35 % v/v)-DMF co-solvent mixture in 62 h. With DMSO as a co-solvent and 5 % imidazole, 98.8 % conversion was achieved in 48 h. With 10 and 20 mol % imidazole, 98 % conversion was achieved in 30 and 24 h respectively. When DMF was used as a co-solvent, 5 and 10 mol % imidazole gave 98 and 99 % conversions in 48 h respectively. 20 Mol % imidazole gave about 99 % conversion in 40 h.

Chapter 5 deals with the production of monoglycerides (MG) by esterification of palmitic acid with glycerol using high active biocatalyst formulations of *Candida antarctica* lipase B (CALB). 48 % Monoglyceride (MG) and 1.4 % diglyceride (DG) were produced after 24 h using free CALB. Increasing the enzyme dose or reaction time did not alter the % conversion in any significant way. Next, the same reaction was carried out with cross-linked enzyme aggregates (CLEAs) prepared from CALB with different concentrations of initially added water in the reaction mixture. It was found that the best result was obtained with 1% water (added initially). 40 % Monoglyceride was formed after 24 h. When same reaction was carried out in the presence of molecular sieves, the % monoglyceride formed was 66 % (in 24 h) in

reaction medium with 1 % (v/v) water content. It was found that as the water content in the reaction mixture increased, monoglyceride content increased from 35 % (when no water was added in the reaction medium) to 66 % (in a medium with 1 % v/v water content). With further increase in water content, there was a decrease in both MG and DG content. Reaction was found to slow down at around 8 h. Assuming that excess water generated during the reaction could be the major cause, second instalment of molecular sieves was added after 8 h. This resulted in the increase in the conversion to 75 % monoglyceride and 10 % diglyceride in 24 h. Continuing with this strategy, 3rd instalment of molecular sieves was added after 12 h which resulted in formation of 81 % monoglyceride. Interestingly, % of diglyceride produced simultaneously went down to 4.5 %. With protein coated microcrystals (PCMCs) prepared using K_2SO_4 as a core material, 70 % monoglyceride and 4 % diglyceride was produced in about 24 h. Cross-linked protein coated microcrystals (CLPCMCs) prepared with 200 mM glutaraldehyde concentration (with K_2SO_4 as a core material) gave about 58 % monoglyceride. When water content was varied in the reaction medium in case of PCMCs catalysed reaction, production of monoglyceride increased from 70 % (when no water was there in the reaction medium) to 82 % (in medium with 0.5 % v/v water content) whereas there was no increase in % of diglyceride. When water content was varied in the reaction medium in case of CLPCMCs (prepared with 200 mM glutaraldehyde concentration) catalysed reaction, production of monoglyceride increased from 58 % (when no water was there in the reaction medium) to 82 % (in medium with 0.5 % v/v water content). On further increasing the water content to 1 %, monoglyceride formed was 87 % whereas diglyceride was just 3.3 %. In reaction medium with 2 % (v/v) water content, 72.5 % monoglyceride and 10.9 % diglyceride

were formed after 24 h. After 48 h, monoglyceride increased to 78.9 % whereas diglyceride decreased to 2.1 %.

Chapter 6 deals with the use of rice bran lipase to carry out biodiesel preparation from rice bran oil and alkaline lipase from *Burkholderia cepacia* strain, ATCC 25609 to carry out some of the transformations. Lipase from rice bran (source which is abundantly available) was used for biodiesel production from rice bran oil. The lipase was extracted from defatted rice bran. The yield of lipase was 5U/10 gm defatted rice bran. The crude lipase was converted to enzyme precipitated and rinsed with acetone preparation (EPRA) and PCMCs (with K_2SO_4 as a core material) and these formulations were used to form biodiesel. Effects of solvent, temperature and water on biodiesel formation were studied. After 24 h, 46 % conversion was obtained with PCMCs of rice bran lipase at 37 $^{\circ}C$ and under nearly anhydrous conditions. When enzyme precipitated and rinsed with acetone (EPRA) preparation of crude extract of *Burkholderia cepacia* lipase was used to carry out transesterification of ethyl butyrate and butanol, 38 % conversion was obtained in about 36 h. Using protein coated microcrystals (PCMCs) of crude extract (with K_2SO_4 as a core material), 76 % biodiesel (from Jatropha oil) was obtained in 48 h. With PCMCs of the purified enzyme, 90 % biodiesel was obtained in about 24 h in solvent free conditions. There was no effect of addition of solvent on the conversion time.

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