6. SUMMARY

The main objective of the thesis was to create designer 'whole-cell biocatalysts' for developing a highly efficient process for the production of enantiopure ethyl (*S*)-4-chloro-3-hydroxybutanoate (ECHB) in high yield. Another objective was to test the developed whole-cell biocatalyst system for the production of industrially important chiral alcohols.

(*S*)-ECHB is a key chiral intermediate in the enantioselective synthesis of Statins. Statins or HMG-CoA reductase inhibitors are a class of potent cholesterol-lowering drugs, making them important in the prevention of coronary heart disease, with total revenues exceeding $25 billion in 2009. The market share of the intermediate (*S*)-ECHB is estimated to be about $ 2 billion.

Synthesis of (*S*)-ECHB is of immense industrial interest and biocatalytic ketone reduction is an attractive alternative to drastic chemical methods, not only in terms of environmental concerns but also in economics of the product formation. Several industries like Bristol–Myers Squibb (USA), Degussa AG (Germany), JFC, Kyoto university and Kaneka corporation have been investigating the application of biocatalysts for synthesis of (*S*)-ECHB. Reduction of ethyl 4-chloro-3-oxobutyrate (ECOB) using whole-cell biocatalysts is particularly interesting because of easy availability of starting material at reasonably low price.

The currently used whole-cell biocatalyst systems suffers from drawbacks such as low efficiency due to barrier imposed by plasma membrane on substrate uptake and product efflux and consequently the complex kinetics of the overall process. To overcome the major drawback of whole-cell system, we proposed to express these enzymes on the surface of cells, i.e. freely hanging in the media but anchored to the membrane. An enzyme expressed in such a manner is expected to behave like a pure, immobilized enzyme, thereby obviating the need for cost-intensive isolation, purification and stabilization of the enzyme. Moreover, kinetics is expected to be much simpler because of the fact that substrate uptake and product efflux across plasma membrane would not be a limitation any more.

Carbonyl reductase (crs1) from *Candida magnoliae* was chosen as enzyme for asymmetric reduction of ketones, which requires NADPH as cofactor. Glucose dehydrogenase (gdh) from *Bacillus megaterium* was selected as enzyme of choice for

in situ cofactor recycling. *E. coli* was chosen as host-cell for cloning and expression of enzymes. The ultimate aim was to co-express both crs1 and gdh together on the surface of *E. coli* cells. However, *a priori* it was not possible to predict whether or not the surface expressed crs1 and gdh would adopt native like confirmation and remain in active form. Therefore, as a first step we attempted expression of both crs1 and gdh separately on the surface of cells in order to test the feasibility of the proposed study.

Thus, we created recombinant *E. coli* strains expressing crs1 and gdh separately on the surface of *E. coli*. The corresponding *E. coli* strains expressing these proteins in cytoplasm were also synthesized for comparison. The expression of protein in recombinant strains was confirmed by SDS-PAGE. The surface expression of crs1 was confirmed by EM immunogold labeling studies. That the crs1 expressed on surface exists in dimeric form, similar to native crs1 expressed in cytoplasm was confirmed by *in vivo* cross-linking of crs1 sub units with glutaraldehyde.

Surface expression level of crs1 and gdh was found to be 17.9-fold and 13.8-fold, respectively lower compared to intracellular expression level of these proteins. However, recombinant *E. coli* strain expressing crs1 on surface showed 15.7-fold higher activity than recombinant strain expressing crs1 intracellularly. Thus, activity per unit protein for recombinant strain expressing crs1 on surface was 275-fold higher compared to recombinant strain expressing crs1 intracellularly. Similarly, the activity per unit protein for gdh in recombinant *E. coli* strain was 225-fold higher compared to recombinant strain expressing gdh intracellularly.

Next, we synthesized *E. coli* strain co-expressing both crs1 and gdh on the surface of cells. crs1 and gdh activity in this strain was found to be 80.51 x 10^3 nmol/min/gm dry cell weight and 152.85 x 10^3 nmol/min/gm dry cell weight, respectively. *E. coli* expressing only crs1 on the surface showed 122.66 x 10^3 nmol/min/gm dry cell weight, which is about 1.5-fold higher than the strain co-expressing both crs1 and gdh on the surface of the cells. Similarly, the strain expressing only gdh on the surface of the cells exhibited activity of 425 x 10^3 nmol/min/gm dry cell weight, which is about 2.8-fold higher than the strain co-expressing both crs1 and gdh. In the controls, *i.e.* strains containing only the corresponding plasmid without the gene for crs1 or gdh did not show any activity, which confirmed that activity is due to the inserted gene in *E. coli* BL21(DE3).

The surface area of *E. coli* is limited. Both crs1 and gdh compete for this limited surface in the strain which co-expresses both of them together, whereas such competition is absent in the strain expressing either crs1 or gdh. However, the *E. coli* strain co-expressing crs1 and gdh on surface of the cells as fusion protein with omp showed 11.4-fold higher activity compared to recombinant *E. coli* strain expressing crs1 within the cells. Similarly, gdh activity in the strain co-expressing both crs1 and gdh on the surface was 9.2-fold higher compared to strain expressing gdh in the cytoplasm. Thus, the designer whole-cell biocatalyst co-expressing both crs1 and gdh is suitable for the production of (*S*)-ECHB.

Yet another important feature from process point of view is that the concentration of NADPH should never become limiting for efficient conversion of ECOB to (*S*)-ECHB. This is possible only when the enzyme responsible for recycling of cofactor by converting $NADP^+$ to NADPH should have higher activity than the enzyme responsible for conversion of ECOB to (*S*)-ECHB. The gdh activity was about 1.9-fold higher than crs1 activity in strain co-expressing both crs1 and gdh, which is sufficient for efficient recycling of cofactor.

In order to improve expression levels of crs1 and gdh in strain co-expressing both the protein on the surface of cells, we expressed crs1 and gdh fusion protein, in which crs1 and gdh were separated by various linkers. omp tag was added for surface expression. We screened several linkers, and successfully expressed protein up to the size of 75 KDa in *E. coli*. Although, some of these construct did show some activity, the strategy proved to be unsuccessful for the purpose of creating an efficient whole-cell biocatalyst system.

In literature certain strains of *E. coli*, the so called Walker strains have been reported. These strains have been extensively used for display of a variety of membrane proteins. We expressed crs1 and gdh, either separately or together in Walker strains *E. coli* C41(DE3), *E. coli* C43(DE3), *E. coli* C41(DE3)pLysS and *E. coli* C43(DE3)pLysS. *E. coli* strain C41(DE3) was found to be optimal amongst all strains tested for surface expression of crs1 and gdh, either separately or together. Strain C41(DE3) co-expressing crs1 and gdh exhibited 2.2-fold higher activity for crs1 and 3.4-fold higher activity for gdh, when compared to strain BL21(DE3). More importantly, gdh activity was 2.92-fold higher than crs1 activity in recombinant *E. coli* C41(DE3). This is highly desirable property from the process point of view as the

concentration of co-factor NADPH required for conversion of ECOB to (*S*)-ECHB will never become limiting because of its efficient recycling by gdh enzyme.

Thus, recombinant *E.coli* C41(DE3) + *pETDuet1-omp-crs1,omp-gdh* may be regarded as highly efficient 'designer whole cell biocatalyst' for conversion of ethyl 4-chloro-3-oxobutyrate to industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate.

Finally, the developed strains *E. coli* BL21(DE3) + *pET 23(a)-omp-crs1*, *E. coli* BL21(DE3) + *pET 29(a)-omp-gdh*, *E. coli* BL21(DE3) + *pETDuet-omp-crs1, omp-gdh* and *E. coli* C41(DE3) + *pETDuet-omp-crs1,omp-gdh* were tested for gram scale production of (*S*)-ECHB using bi-phasic reaction medium. All the strains were found to be quite efficient biocatalysts for the production of (*S*)-ECHB. The strain expressing only crs1 on surface gave productivity of 13.59 g/l/hr/g dry cell weight. It was increased to 45.11 g/l/hr/g dry cell weight, when recombinant strain co-expressing both enzyme was used as biocatalyst. This 3.32-fold increase in the productivity came as a pleasant surprise to us. The most probable explanation for this increased activity could be that the cofactor NADPH/$NADP^+$ does not become completely free in solution; instead, it gets channelized between crs1 and gdh which are localized in close proximity to each other on the surface of cells. The productivity with *E. coli* Walker strain C41(DE3) was about 1.5-fold higher compared to corresponding strain BL21 (DE3). This was expected, as the crs1 activity of C41(DE3) was about 1.6-fold higher than BL21(DE3) strain.

Further, we tested the 'designer whole-cell biocatalyst' for the asymmetric reduction of a range of alkyl and aryl ketones. The recombinant strain expressing crs1 on the surface of cells showed 50 to 228-fold higher activity per unit protein compared to recombinant *E. coli* strain expressing crs1 intracellularly. A range of industrially important chiral alcohols were prepared in >99% e.e.