

CHAPTER-VI
SUMMARY AND CONCLUSION

Enzymes are the subject of wide investigation both for understanding the basic mechanism of action and also to exploit its catalytic potential for various applications. The use of whole cells as biocatalysts in the place of purified soluble enzymes is an interesting alternative which is gaining recognition in recent years. Although the cell membrane permeability barrier is a major problem encountered while using whole cells, this can be alleviated by the use of various permeabilizing agents.

Several intracellular enzymes from Baker's yeast (*Saccharomyces cerevisiae*) such as alcohol dehydrogenase, hexokinase and glucose-6-phosphate dehydrogenase are commercially available and are being used as analytical and preparative biocatalysts. Intact whole cells of Baker's yeast showed very low activities of these intracellular enzymes, mainly due to the cell membrane permeability barrier. Baker's yeast cells were treated with a number of known permeabilizing agents, among which the cationic detergent cetyltrimethylammonium bromide (CTAB) permeabilized the cells to the greatest extent. Treatment with CTAB resulted in an increase in intracellular enzyme activities. A comparative study of the properties of ADH within CTAB permeabilized Baker's yeast cells with those of the enzyme isolated from the same organism was carried out. Permeabilized Baker's yeast cells were also used as a biocatalyst for various purposes. The results of this study are summarised below.

1. Intact Baker's yeast cells exhibited very low activities of HK, G6PDH and ADH. Treatment of the cells with CTAB resulted in an increase in enzyme activity ranging from 7 to 580 fold depending on intracellular contents of various enzymes. The ADH activity in CTAB permeabilized cells was about 40% higher than that of cell-free extract prepared by toluene autolysis.
2. Maximum permeabilization was obtained when the cells were treated with 0.2% CTAB at 25°C for 30min.
3. ADH within permeabilized cells exhibited a maximum activity at pH 9.5 whereas the pH optimum of purified enzyme was found to be pH 9.0. The enzyme in permeabilized cell exhibited a K_m value of $13.3 \times 10^{-3} M$ for ethanol and $2.2 \times 10^{-4} M$ for NAD^+ . These values were almost similar to those for purified enzyme.
4. Purified ADH is stable at pH 7.0 and gets inactivated at acidic and alkaline pH ranges whereas the enzyme within permeabilized cell was found to be stable over a broad range of pH (pH 5.5-10.0). The enzyme within permeabilized cell also showed increased thermal stability. For example, its half-life at 65°C was 2.5 h whereas that for purified enzyme was only 15 min.
5. The enzyme within permeabilized cell was also more stable against denaturing agents such as urea and guanidine hydrochloride and proteases such as trypsin, chymotrypsin and papain.

6. Alcohol dehydrogenase was found to leak out of the permeabilized cells stored in buffer. Attempts to prevent this leakage with glutaraldehyde treatment resulted in partial success. When whole cells of Baker's yeast were treated with 0.1% glutaraldehyde for 10 min at 4°C, although an initial loss of 30% in enzyme activity was observed (due to inactivation by glutaraldehyde) there was no further decrease in enzyme activity after permeabilization and storage of cells.
7. CTAB permeabilized Baker's yeast cells were used as a source of .ADH for the reduction of cinnamaldehyde to cinnamyl alcohol in aqueous system. The pH optimum of the reaction was found to be pH 7.0. The K_m value for cinnamaldehyde was $1.0 \times 10^{-4} M$ and that for NADH was $0.59 \times 10^{-4} M$.
8. Due to the poor solubility of cinnamaldehyde and cinnamyl alcohol in aqueous system, the reaction was performed in organic solvent media. CTAB permeabilized cells were supplemented with NAD^+ , immobilized on glass beads and used as a biocatalyst for the conversion in organic media.
9. Di-isopropyl ether was found to be the most suitable for performing bioconversion of cinnamaldehyde to cinnamyl alcohol.
10. The repeated use of the biocatalyst resulted in a decrease in bioconversion and also desorption of cells from the glass beads.

11. Baker's yeast cells were also permeabilized using various organic solvents among which acetone permeabilized cells exhibited maximum cellular ADH activity.
12. Cells prepared by the conventional procedure of acetone treatment required the exogenous addition of cofactors for increased production of cinnamyl alcohol. A modification over this procedure was developed by freezing the Baker's yeast cells into a pellet, prior to treatment with acetone. Maximum bioconversion of cinnamaldehyde was achieved using these cells even without the exogenous addition of nicotinamide cofactor.
13. The rate of the reaction was not affected by altering the pH of the buffer used to saturate di-isopropyl ether (bulk-phase pH). This is because the chemical state of an enzyme remains unchanged from that of aqueous solution from which it was last recovered (pH memory).
14. The half-life of ADH in acetone permeabilized cells suspended in di-isopropyl ether was much higher (half-life at 55°C is 40 h) than those suspended in sodium phosphate buffer (half-life at 55°C is 5 h). An increase in the rate of conversion of cinnamaldehyde to cinnamyl alcohol was observed on increasing the reaction temperature from 25°C to 45°C.
15. A complete conversion of 50 μ moles of cinnamaldehyde was obtained when 150 mg of acetone permeabilized cells were suspended in di-isopropyl ether at 37°C for 8 h. After the reaction, the biocatalyst was easily removed by simple filtration or centrifugation. No further extraction and purification of the product cinnamyl alcohol was necessary.

16. Maximum bioconversion of cinnamaldehyde was obtained using acetone permeabilized Baker's yeast cells. However, on repeated use of the biocatalyst, a gradual decrease in the conversion with each cycle was observed, the conversion in 4th cycle being just 14%. This was shown to be due to the partial loss of cofactors, since addition of NAD^+ in each cycle reverted this decrease.
17. CTAB permeabilized Baker's yeast cells were used as a source of ADH and G6PDH, in the place of the isolated enzymes, for the preparation of reduced nicotinamide cofactors NADH and NADPH, respectively.
18. The reduced nicotinamide cofactors were obtained as a fine powder and were found to be as pure as the authentic samples by both TLC and HPLC.
19. Permeabilized Baker's yeast cells were used as a single source of either ADH or HK/G6PDH for the estimation of ethanol or glucose, respectively. The method using permeabilized cells was used for the estimation of ethanol and glucose in fermented broth and blood samples, respectively. The results correlated well with those obtained using purified ADH and glucose oxidase/peroxidase methods which are the standard methods of ethanol and glucose estimation.

Thus, the permeability barrier for the movement of substrate and/or products across the cell membrane was removed by treating the cells with CTAB. The CTAB treatment developed to prepare permeabilized cells is simple, rapid and easy to scale-up. These permeabilized cells can be used as a suitable system for studying the behaviour of enzymes *in vivo* and also as an efficient biocatalyst for varied applications.

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