DEVELOPMENT OF IMMOBILIZATION AND DRYING METHODS OF ENZYMES ON SUPPORT PARTICLES FOR ENZYMATIC GAS-PHASE REACTIONS

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der Rheinisch-Westfälischen Technischen Hochschule Aachen zur Erlangung des Akademischen Grades eines Doktorin der Naturwissenschaften genehmigte Dissertation

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ABSTRACT

In this research work, two mesophilic alcohol dehydrogenases namely baker’s yeast alcohol dehydrogenase (YADH) and *Lactobacillus brevis* alcohol dehydrogenase (LBADH) and one thermophilic alcohol dehydrogenase namely *Thermoanaerobacter* species alcohol dehydrogenase (ADH T) were immobilized by physical adsorption method. The effects of various immobilization and drying process parameters on the residual activity and the protein loading of the immobilized enzyme preparation were studied and thereby different optimum preparations were observed for different enzymes. Under the optimum immobilization conditions the residual activity achieved with YADH, LBADH, and ADH T was about 80 %, 316 %, and 325 %, respectively. The hypothesis of bubble nucleation as a cause for loss of enzyme activity during the low pressure drying process was verified. The effects of various gas-phase reaction conditions on the initial reaction rate and the half-life of the optimized preparations were also studied. It was observed that addition of a suitable buffer (50 mM phosphate buffer, pH 7) or an optimum amount of sucrose (5 times greater than the amount of protein on weight basis) during the enzyme immobilization enhanced the half-life of the immobilized enzymes in the gas-phase reaction. Water activity significantly influenced the initial reaction rate and the half-life of the immobilized enzyme preparations in the gas-phase reaction. The optimum water activity found for LBADH and ADH T was the same (0.55). Under the optimized immobilization and gas-phase reaction conditions the thermo-stability of the ADH enzymes was enhanced tremendously. The space-time yield of *(R)*-phenylethanol was about 1000 gm⋅l$^{-1}$⋅d$^{-1}$ with LBADH and the space-time yield of *(S)*-phenylethanol was about 600 gm⋅l$^{-1}$⋅d$^{-1}$ with ADH T. The total turnover number of LBADH was about $9\times10^5$ and the same of ADH T was about $3\times10^5$. 
DEDICATED TO MY FAMILY
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I experience great pleasure to convey my profound sense of gratitude and respect to Prof. Dr.-Ing. Jochen Büchs, the chair of Biochemical Engineering of RWTH-Aachen, for his excellent supervision, encouraging discussions, and inspiring suggestions.

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Every wheel needs an axis to keep on rotating. In my case, my axis was and it is my parents – source of my all inspirations. They shouldered the burdens of every little problem just to make me successful in this work. There were also my brothers, sister-in laws, Abhishek, divyanshu, and my friends Manisha and Dipayan who played very significant roles on different aspects of my life through out my stay in abroad. Any attempt to thank them would defy the greatness of their contributions.

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CURRICULUM VITAE
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<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>ADH T</td>
<td><em>Thermoanaerobacter</em> species alcohol dehydrogenase</td>
</tr>
<tr>
<td>APM</td>
<td>absorbance per minute, it is expressed in min(^{-1})</td>
</tr>
<tr>
<td>DF</td>
<td>dilution factor, it is a dimensionless quantity</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>HLADH</td>
<td>horse liver alcohol dehydrogenase</td>
</tr>
<tr>
<td>HT</td>
<td>heat-treated cell extract</td>
</tr>
<tr>
<td>IU</td>
<td>international unit, it is expressed in (\mu)mol(\cdot)min(^{-1})</td>
</tr>
<tr>
<td>LBADH</td>
<td><em>Lactobacillus brevis</em> alcohol dehydrogenase</td>
</tr>
<tr>
<td>Mes</td>
<td>2-((N\text{-morpholino}))ethane sulfonic acid</td>
</tr>
<tr>
<td>Mops</td>
<td>3-((N\text{-morpholino}))propane sulfonic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAD(^+)</td>
<td>nicotinamide adenine dinucleotide; oxidized form</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide; reduced form</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADP(^+)</td>
<td>nicotinamide adenine dinucleotide phosphate; oxidized form</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate; reduced form</td>
</tr>
<tr>
<td>P</td>
<td>plain cell extract</td>
</tr>
<tr>
<td>PEEK</td>
<td>polyetheretherketone</td>
</tr>
<tr>
<td>PL</td>
<td>path length, it is expressed in cm</td>
</tr>
<tr>
<td>PO(_4)</td>
<td>potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>POM</td>
<td>polyoxymethylene</td>
</tr>
<tr>
<td>READH</td>
<td><em>Rhodococcus erythropolis</em> alcohol dehydrogenase</td>
</tr>
<tr>
<td>RH</td>
<td>relative humidity, it is expressed in %</td>
</tr>
<tr>
<td>STY</td>
<td>space-time yield, it is expressed in gm(\cdot)l(^{-1})(\cdot)d(^{-1})</td>
</tr>
<tr>
<td>SV</td>
<td>sample volume, it is expressed in ml</td>
</tr>
<tr>
<td>TBADH</td>
<td><em>Thermoanaerobium brockii</em> alcohol dehydrogenase</td>
</tr>
<tr>
<td>Tea</td>
<td>triethanolamine hydrochloride</td>
</tr>
<tr>
<td>TEADH</td>
<td><em>Thermoanaerobacter ethanolicus</em> alcohol dehydrogenase</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
<tr>
<td>TTN</td>
<td>total turn over number, it is a dimensionless quantity</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
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<tr>
<td>TV</td>
<td>total volume, it is expressed in ml</td>
</tr>
<tr>
<td>YADH</td>
<td>baker’s yeast alcohol dehydrogenase</td>
</tr>
<tr>
<td>$a_w$</td>
<td>water activity, it is dimensionless</td>
</tr>
<tr>
<td>$a_X$</td>
<td>thermodynamic activity of compound X, it is dimensionless</td>
</tr>
<tr>
<td>$g$</td>
<td>relative centrifugal force, it is dimensionless</td>
</tr>
<tr>
<td>$P_{psat_X}$</td>
<td>saturation vapor pressure of compound X, it is expressed in atm (1 atm = 101 325 Pa)</td>
</tr>
<tr>
<td>$P_{p_X}$</td>
<td>partial pressure of compound X, it is expressed in atm</td>
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<tr>
<td>$\varepsilon$</td>
<td>reduced cofactor extinction coefficient of a reduced cofactor, it is expressed in ml·µmol⁻¹·cm⁻¹</td>
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PREFACE

This dissertation is submitted to Faculty of Mathematics, Computer Sciences, and Natural Sciences in RWTH-Aachen University, Germany, in partial fulfillment of the requirements for the degree of “Doktor rerum naturalium (Dr.rer.nat.)”. It is divided into five chapters.

Chapter 1 introduces the topic of the dissertation. It discusses the importance and background of this topic. Also the objectives of this research work are briefly presented.

Chapter 2 is based on a review of literature related to this research work. It mainly covers about what is known till date regarding an enzyme immobilization and an enzymatic gas-phase reaction.

Chapter 3 tells about the materials used in this research work. Also the methods of immobilized enzyme preparation and their evaluation are discussed. Additionally, the experimental set-up for batch and continuous gas-phase reaction is reported.

Chapter 4 covers the results obtained in this research work with relevant discussion on them. The previous findings of other researchers’ in relation to those results are also cited.

The final chapter, Chapter 5, summarizes the results and proposes new research directions for the future.

In this dissertation, the Bibliography follows the style of citations of Biotechnology and Bioengineering journal.

The Appendix mainly contains essential information, which, for brevity, were not included in the main text.
1 INTRODUCTION
Biocatalysis has been a key focus area in white biotechnology (application of nature’s toolset to industrial production). A recent report of McKinsey predicted that by the year of 2010, white biotechnology would be a competitive way of producing about a fifth of world’s chemical output (equivalent to US$ 160 billion) (Bachmann, 2003). This is because of the growing use of enzymes to replace conventional inorganic catalyst in production processes. In fine chemical segment, revenues from products being manufactured using biotechnology will increase from 15% at start of the millennium to approximately 30% to 60% by 2010 (Bachmann, 2003). Since many chemical and biochemical transformations involve oxidation/reduction processes, developing practical biocatalytic applications of oxidoreductases has long been an important goal in biotechnology. In particular, the alcohol dehydrogenase (ADH) enzymes are being increasingly used for asymmetric synthetic transformations, fuelled by the growing demand for enantiopure pharmaceuticals (Ferloni, et al. 2004; Hummel, 1999; Wong and Whitesides, 1994 and Bradshaw, et al. 1992), biosensors (Hummel and Kula, 1989; Williams and Hupp; 1998 and Jones, 1986), and for decomposition of pollutants especially volatile organic compounds (Goubet, et al. 2002 and Maugard, et al. 2001).

But as long as the use of ADH enzymes is restricted to the aqueous reaction medium, the scope of industrial bioconversions, especially for the production of fine chemicals, is necessarily limited by a variety of constrains: the operational instability of ADH enzymes and cofactors (NAD$^+$ or NADP$^+$) (Olrich, et al. 2002; Walt, et al. 1984; Ooshima, et al. 1981; Wong and Whitesides, 1981 and Jones and Beck, 1976), insolubility of many of the substrates and the products in water (Gröger, et al. 2002), product inhibition of the enzyme (Lee and Whitesides, 1985), lack of stability of some substrates and products in aqueous solutions (Keinan, et al. 1986b), and difficulty of product and enzyme recovery from aqueous medium. In principle, most of these problems might be overcome by switching from water to organic solvents as reaction medium. Nevertheless, the toxicity of the organic solvents to the enzyme and environmental pressure forces the application of other non-conventional medium which should be non-toxic, non-hazardous, readily separated from the enzyme and the product. Therefore, an attention is currently being paid to the use of gas-phase catalysis for biotransformations (Goubet, et al. 2002; Maugard, et al. 2001 and Robert, et al. 1992).
Most of the earlier studies in the gas-phase catalysis were conducted using lyophilized cells or enzymes (Yang and Russell, 1996b; Pulvin, et al. 1986; Kimura, et al. 1979 and Yagi, et al. 1969). On the other hand, almost all the recent studies in the gas-phase catalysis are performed using immobilized enzymes (Ferloni, et al. 2004; Létisse, et al. 2003; Pires et al. 2002; Lamare and Legoy, 1995b; Kim and Rhee, 1992; Ross and Schneider, 1991 and Barzana, et al. 1987). These authors have reported that the application of lyophilized powder for gas-phase catalysis results in some problems which include: aggregation of the lyophilized powder even at low water activity (mainly for non-purified enzymes) (Ferloni, 2004; Parvaresh, et al. 1991 and Lamare and Legoy, 1995b), shrinkage and channeling of the packed bed at high temperature (Yang and Russell 1996b), undefined structure and mass transfer, and lack of thermo-stability for long time continuous process (Goubet, et al. 2002). Immobilization of cells or enzyme could solve some of the problems associated with lyophilized cells or enzymes in a gas-phase reaction system. Although immobilization of whole cells with in-situ cofactor regeneration may alleviate the problem of addition of expensive cofactor, but there are reports of a decrease in enzyme activity in a gas-phase reaction due to the incomplete regeneration of cofactor using whole cells (Goubet, et al. 2002), low diffusion of gaseous substrates to the enzyme due to the presence of cell wall and membranes, and poor selectivity resulting in by-product formation (Grizon, et al. 2004). Thus, immobilization of the enzyme with its cofactor is advantageous for continuous operations. In addition, it also enhances the thermo-stability of the ADH enzymes (Liao and Chen, 2001 and Julliard, et al. 1986).

Enzyme immobilization by physical adsorption is the most simple and widely used technique for gas-phase catalysis (Ferloni, et al. 2004; Létisse, et al. 2003; Lamare and Legoy, 1995b and Barzana, et al. 1987). It is known that several immobilization process parameters such as addition of sucrose (Ferloni, et al. 2004) or the hygroscopicity of buffer salt used during enzyme immobilization influence the activity and the thermo-stability of the enzyme in gas-phase reaction (Ross and Schneider, 1991). However, no systematic study has been conducted till date to optimize the adsorptive immobilization of ADH enzymes in order to use them efficiently in gas-phase reaction. In this research work, two mesophilic ADH enzymes namely yeast alcohol dehydrogenase (YADH) and Lactobacillus brevis alcohol dehydrogenase (LBADH) and one thermophilic ADH enzyme namely Thermoanaerobacter species alcohol dehydrogenase (ADH T) were
Chapter 1  Introduction

immobilized by physical adsorption method. The effects of various immobilization and
drying process parameters on the residual activity and the protein loading of the
immobilized enzyme preparations were studied. The influence of various
immobilization process parameters on the initial reaction rate and the half-life of the
immobilized enzyme preparations in gas-phase reaction was evaluated. Optimization of
the gas-phase reaction parameters for production of (R)- and (S)-phenylethanol using
LBADH and ADH T, respectively, was done. Steps were also taken for the development
of a suitable batch gas-phase reactor in order to run many gas-phase reactions in
parallel, which would aid in obtaining more information within short time at the cost of
less energy.
2 REVIEW OF LITERATURE
2.1 Alcohol dehydrogenase enzymes

Alcohol dehydrogenase (ADH) is an oxidoreductase enzyme, requiring either NADH or NADPH as a coenzyme, which reacts with primary and secondary, linear and branched-chain, aliphatic and aromatic alcohols with their corresponding aldehydes and ketones. Most of the ADH enzymes are of limited use for technical applications because of their lack of substrate specificity (yeast alcohol dehydrogenase (YADH), horse liver alcohol dehydrogenase (HLADH), *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH), and *Thermoanaerobacter ethanolicus* (TEADH)), narrow substrate spectrum (YADH, HLADH, TBADH, and TEADH), poor operational stability (YADH and HLADH), and low enantioselectivity (YADH and HLADH) (Keinan, et al. 1986a and Hummel, 1997). Recently, two stereoselective ADH enzymes such as R-specific alcohol dehydrogenase (R-ADH) from *Lactobacillus brevis* (LBADH) (Hummel, 1997) and as S-specific alcohol dehydrogenase (S-ADH) from *Thermoanaerobacter* species (Daussmann and Hennemann, 2004) with high substrate specificity, broad substrate spectrum, high enantioselectivity, and good operational stability are commercially available.

However, the high cost of the nicotinamide cofactors is a limiting factor in large scale application of these ADH enzymes. The application of whole microbial cells with in-situ cofactor regeneration for synthetic purpose could avoid the above mentioned problem, but their selectivity is poor (Grizon, et al. 2004 and Goubet, et al. 2002) and the space-time yield is low as compared to the isolated enzymes. Hence, the in-situ regeneration of this stoichiometrically used amount of the cofactor during an enzymatic reaction is necessary. Generally, a cofactor regeneration method must be capable of recycling the cofactor $10^2 - 10^6$ times to be economic, depending on the initial cost of the cofactor and the value of the product synthesized (Donk and Zhao, 2003). Usually enzymatic methods for cofactor regeneration are used because they are specific for the regeneration of enzymatically active cofactor and are mostly compatible with other components of an enzymatic reaction. The enzymatic method with a coupled second enzyme for regeneration has been developed using formate dehydrogenase from *Candida boidinii* for the regeneration of NADH (Yamazaki and Maeda, 1982 and Wichmann, et al. 1981). However, the enzyme is expensive when purchased commercially. A few formate dehydrogenase enzymes from *Clostridium thermoacticum*
and *Pseudomonas* species for NADPH regeneration are known (Seelbach, et al. 1996 and Wong and Whitesides, 1981), but up to now they find application only in lab scale. Other options include the use of glucose dehydrogenase or glucose 6-phosphate from *Leuconostoc mesenteroides* for NADPH regeneration (Wong and Whitesides, 1981). According to Wong and Whitesides, (1981) the ADH enzyme and its cofactor are significantly unstable in solution.

Application of enzymes and cofactors in gas-phase reaction medium with restricted water availability would minimize this stability problem. But due to the non-volatility of the second substrate, the regeneration of cofactor using a second enzyme is not possible with glucose dehydrogenase. ADH enzyme catalyzed gas-phase reactions, substrate coupled cofactor regeneration is usually carried out using the same enzyme with isopropanol and ethanol as cosubstrate for regeneration of NADPH and NADH cofactors, respectively. Inevitably, the coupled substrate approach suffers from equilibrium limitations.

The characteristic features of the alcohol dehydrogenase enzymes such as yeast alcohol dehydrogenase (YADH), *Lactobacillus brevis* alcohol dehydrogenase (LBADH), and *Thermoanaerobacter* species alcohol dehydrogenase (ADH T) are described below.

### 2.1.1 Yeast alcohol dehydrogenase

Yeast alcohol dehydrogenase (YADH; E.C. 1.1.1.1) from baker’s yeast is a tetrameric enzyme with 8 zinc ions. YADH has a molecular weight of 141 kDa. The active site at each subunit contains one zinc ion, which is absolutely necessary for enzyme activity (Coleman and Weiner, 1973). The second zinc ion (conformational zinc) on each subunit of the enzyme plays a prominent role, probably by stabilizing the tertiary structure of YADH (Magonet, et al. 1992). It is known that the sulfhydryl groups (Cys-46 and Cys-174) located in the active site are essential for enzyme activity and are bound to the catalytic zinc ion (Coleman and Weiner, 1973). The enzyme is stable at pH between 6 and 8; at temperatures below 50 °C. The enzyme requires NADH as a coenzyme and has a narrow specificity for primary alcohols or aldehydes.
2.1.2 *Lactobacillus brevis* alcohol dehydrogenase

The NADPH-dependent R-ADH from *Lactobacillus brevis* (LBADH; E.C.1.1.1.2) is a tetramer of four identical subunits each of which consists of 251 amino acids. LBADH has a molecular weight of 106 kDa. The enzyme contains both zinc and magnesium ions. The enzyme is stable at pH between 6 and 9; at temperatures below 60 °C (Riebel, 1996). The enzyme catalyzes the reduction of ketones, keto such as alpha, beta or gamma ketone esters, and cyclic aryl and alkyl esters preferably those with residues substituted by halogen or alkyl groups (Hummel, 1997).

2.1.3 *Thermoanaerobacter* species alcohol dehydrogenase

The NADPH-dependent S-ADH from *Thermoanaerobacter* species (ADH T) is supposed to be a tetrameric enzyme containing zinc in its active site. ADH T has a molecular weight of 149 kDa. The temperature stability of the enzyme is in the range of 30 °C to 70 °C. The maximum enzyme activity in solution is obtained at pH 7. The isoelectric point is reported to be 5. The enzyme is known to catalyze reduction of both aliphatic and aromatic ketones, diketones, aldehydes and 2- and/or 3-ketone esters (Daussmann and Hennemann, 2004).

2.2 Definition of immobilized enzymes

Immobilized enzymes are defined as “enzymes which are physically confined or localized in a certain region of space with retention of their catalytic activities, and which can be used repeatedly and continuously” (Katchalski-Katzir, 1971).

2.3 Advantages and disadvantages of enzyme immobilization

The main advantages of enzyme immobilization are:

- Low downstream processing cost
- Possibility of enzyme recycling
- Cofactor binding to enzyme (less waste)
- Easy realization of continuous production
The main disadvantages of enzyme immobilization are:

- Loss of absolute enzyme activity due to the immobilization process
- Additional cost of carrier or other reagents used for immobilization process
- Potential for mass transfer limitations

### 2.4 Classification of enzyme immobilization methods

Figure 2.1 shows the classification of the enzyme immobilization method based on the nature of interaction of the enzyme with the support (Chibata, 1978).

![Common enzyme immobilization methods](image)

**Figure 2.1: Common enzyme immobilization methods**

### 2.5 Optimization of enzyme immobilization parameters

Immobilization of enzymes represents an additional step in the biocatalysis. However, this procedure has become an indispensable part of the industrial biotransformations especially useful for expensive enzymes (Liese, et al. 2000). The choice of the immobilization method depends on the enzyme stability (Keinan, et al. 1986a and Kelly,
et al. 1977) and the process of application. The most commonly used immobilization methods are adsorption or covalent linkage to a carrier. Covalent coupling is not necessary in non-conventional media such as gas-phase media, since the proteins are generally insoluble in this media. Moreover, immobilization of enzyme by physical adsorption provides a simple mean for retention in continuous reactors (Hartmeier, 1988 and Chiou and Beuchat, 1986). Often, an increased thermostability of the catalyst after adsorption is observed (Oh, et al. 2000; Martinek, et al. 1977 and Pwereira, et al. 2001). Physical adsorption induces only slight modifications on the conformation of the enzyme as the binding mainly results from hydrogen bonds, salt linkages or van der Waal's forces (Kierstan and Coughlan, 1991). Nevertheless, too strong interaction between the support material and protein could lead to variations on tertiary structure and finally to deactivation of enzyme (Bucholz and Kasche, 1997).


It was reported that the optimum pH of the enzyme is usually shifted after immobilization (Bajpai and Sachdeva, 2002). According to Gitlesen, et al. (1997), the influence of the pH change on the adsorption of lipase is not significant indicating that hydrophobic interactions are the dominating adsorption forces. Bajpai and Sachdeva, (2002), studied the effect of phosphate, chloride, and sulfate salts of different concentrations on the immobilization efficiency of diastase enzyme. They observed that the relative activity of the immobilized diastase enzyme was suppressed at 200 mM salt concentration. According to Tong, et al. (2001), both the electrostatic and the hydrophobic interactions could be affected by ionic strength of a salt. They observed that the adsorption of lysozyme enzyme on the magnetic particles decreases from 71 mg/g to 21 mg/g of the support with the increase in sodium chloride concentration from
0 mM to 0.1 mM. Liao and Chen, (2001), reported that the residual activity of immobilized YADH obtained at 4 °C (62 %) was much higher than that obtained at 25 °C (17 %). According to them, this was due to reduced thermo-inactivation of enzyme at low temperature.

Pretreatment of the organic supports with solvents such as ethanol, propanol, acetone or methanol lead to higher residual activity of lipase after immobilization (Montero, et al. 1993). According to Bailey and Cho, (1983), the specific activity of the immobilized enzyme increases with the decrease in particle size of the supports. Adsorption of proteins on solid surfaces is the net result of various types of interactions that involve the properties of an enzyme molecule, the carrier surface, solvent, and the presence of other solutes (Geluk and Norde, 1994). According to Soderquist and Walton, (1980), structural changes induced during the adsorption increase with decreased surface coverage and this leads to loss of enzyme activity. In this respect, Barros, et al. (1988) reported that low enzyme activity at low enzyme loading is due to direct inactivation of the enzyme by the support, which is probably owing to the enzyme conformational change/unfolding at the support surface. This loss in enzyme activity can be prevented by addition of additives which protect the enzyme during immobilization (Wehtje, et al. 1993). Snijder, et al. (1991) reported, that high residual activity of immobilized YADH is obtained by addition of stabilizers such as bovine serum albumin (BSA) during enzyme immobilization.

In spite of all attempts, still a quite significant loss in enzyme activity during the immobilization by physical adsorption is reported by Montero, et al. (1993). Also the effect of other immobilization parameters such as stirring temperature, stirring time, stirring speed, addition of sucrose, type of buffer, drying temperature, and drying pressure are seldomly studied.

### 2.6 Principle of enzymatic gas-phase reaction

The dry enzyme catalyzes the reaction of gaseous substrate to gaseous product (Figure 2.2). In a continuous reactor, gaseous substrate continuously enters into the reactor, which is usually a packed bed enzyme reactor, where the reaction takes place and the unreacted substrate and the product continuously leave the reactor. An enzymatic gas-
Phase reaction can be operated also in the batch mode. In a batch reactor, the dry enzyme catalyzes the conversion of gas-phase substrates, which are held in the vessel.

![Figure 2.2: Concept of enzymatic gas-phase reaction](image)

### 2.7 Characteristics of enzymatic gas-phase reaction

Enzymatic gas-phase reaction involves conversion of gaseous substrate to gaseous product using dry enzyme as catalyst. This method offers many advantages compared to conventional biocatalysis, which includes (Lamare, et al. 2004; Cameron, et al. 2002; Yang and Russell, 1996b and Ross and Schneider, 1991).

- Substrate and product are in the gaseous phase and the biocatalyst is in the dry state; therefore the immobilization of the enzyme and its cofactor is much simplified. Recovery and recycling of the immobilized enzyme preparation is possible.

- The enzyme and its cofactor in a dry state with controlled water content are more resistant to thermo-inactivation; therefore reactions can be performed at elevated temperature. In addition, the reaction can be performed for longer duration.

- The substrates employed are pure and the addition of solvent can be avoided. Hence, the toxicity of solvent towards the catalyst or the final consumer of the product can be avoided. The amount of by-product is limited.

- Downstream processing is simplified due to the absence of solvent. The gas-phase products and unreacted substrate can be separated and recovered with high purity by fractional condensation.

- Diffusion coefficients of the substrate and the product in the gas-phase are more than $10^3$ times higher than in the liquid-phase (Reid, et al. 1977).

- Volatile, toxic or inhibitory substances can be continuously removed.
Independent variation of thermodynamic activities of the different reaction species is possible. Therefore, the effect of each compound on the activity and the thermostability of the catalyst can be evaluated.

Since the bioreactors are operated at high temperatures, there is no danger of microbial contamination. Nevertheless, there are few limitations to the use of enzymatic gas-phase bioreactor. It is applicable only for low boiling compounds. The main problem for many reactions of potential commercial interest is obtaining the substrates and/or products in the gaseous state.

In this regard, the following two factors need to be accounted.

The first is concerning with two physical parameters. The temperature and the absolute pressure are of vital importance in the gas-phase catalysis. Both of these parameters affect the vapor pressure of the different components of a gas-phase reaction system. The vapor pressure (partial pressure $P_p$) of compound X is defined by the following expression

$$P_{pX} = \left( \frac{n \text{ moles of } X \text{ in gas-phase}}{n \text{ total moles in gas-phase}} \right) \times \text{absolute pressure}$$

(2.1)

Then the thermodynamic activity of compound X can be calculated as follows

$$a_X = \frac{P_{pX}}{P_{psat_X}}$$

(2.2)

where

$a_X$ is the thermodynamic activity of compound X,

$P_{pX}$ is the partial pressure of compound X in the gas entering the bio-reactor,

$P_{psat_X}$ is the saturation vapor pressure of compound X. It is also known that temperature and pressure affects not only the activity of the biocatalyst by influencing the concentration of the compounds in the gas-phase, but also the stability of the biocatalyst.
The second key factor to be considered is the water activity \( (a_w) \). It is defined as ratio of partial pressure of water in the system to the saturation vapor pressure of pure water at the same temperature. Any change in water activity affects the hydration state of the biocatalyst, which in turn influences the activity and the thermo-stability (The thermo-stability is defined in terms of half-life of the catalyst.) of an enzyme in a gas-phase reaction. Previous studies on the effect of water activity revealed that the enzyme activity increases with increase in water activity; but at the same time thermo-stability of the enzyme decreases (Erable, et al. 2004; Goubet, et al. 2002; Yang and Russell, 1996b and Barzana, et al. 1989). This effect of water can be clarified using water adsorption-isotherm curve. At a definite temperature, it is possible to vary the water content (i.e., the hydration state of the enzyme) by changing the water activity of the surrounding gas-phase. The relation between these two variables is schematically shown in Figure 2.3.

![Figure 2.3: Schematic representation of water content versus water activity (adapted from Drapron, 1985): Point O represents the amount of strongly bound water to the protein, point B is the point of appearance of ‘solvent water’ and point C corresponds to the amount of ‘non-solvent’ water.](image)

The water adsorption-isotherm curve has three main parts. The first part (point O to point A) corresponds to low water activity, exhibiting non-linear behavior between the water content of the protein and the water activity. It denotes the interaction of water with ionizable groups on the protein. At point A, the first hydration layer of the water is formed. Then the water content of the protein increases linearly with the water activity until point B. The linear region corresponds to the binding of water to the polar sites on the protein and becomes less structured (Hahn-Hagerdål, 1986). The point B
corresponds to the appearance of free water. On the last part of the isotherm (onwards from point B), a distinct aqueous phase exists, and the water content of the biocatalyst increases dramatically with the water activity. The ‘upswing’ in the plot above point B is due to water condensing onto weakest binding sites on the protein surface to complete the protein hydration process. The point C corresponds to the total quantity of non-solvent water (Drapron, 1985).

Most of the previous studies concerning the effect of water activity on the enzyme activity in gas-phase catalysis showed that a critical water activity is required for the enzyme to become active (Erable, et al. 2004; Grizon, et al. 2004; Goubet, et al. 2002 and Maugard, et al. 2001). The critical water activity varies with the source of the enzyme and its preparation resulting from addition of buffers, sucrose, etc. (Jones, 1986 and Ross and Schneider, 1991). Then the activity of the enzyme in gas-phase increases with increase in water activity. This may be related to an increase in flexibility of the enzyme as water acts as a plasticizer (Hartsough and Merz, 1993; Klibanov, 1989 and Zaks and Klibanov, 1988). The drastic decrease in thermo-stability of the enzyme after a specific water activity might be owing to the thermo-inactivation of enzyme mediated by water (Turner, et al. 1995; Rupley and Careri, 1991; Klibanov and Ahern, 1987; Klibanov, 1986 and Kuntz and Kauzmann, 1974).

It was observed by Erable, et al. (2005); Ferloni, et al. (2004) and Goubet, et al. (2002) that with the increase in temperature of a gas-phase reaction, the activity of an enzyme increased, but at the cost of decrease in the thermo-stability of the enzyme. In addition, various studies are known, which investigated several other parameters such as the total gas flow rate (Erable, et al. 2004 and Lamare, et al. 1997), substrate activity (Goubet, et al. 2002 Maugard, et al. 2001 and Hwang and Park, 1997), amount of added enzyme (Barzana, et al. 1989 and Hwang and Park, 1997) on the enzyme activity in a gas-phase reaction system.


3 MATERIALS, METHODS, AND EXPERIMENTAL SET-UP
3.1 Materials

3.1.1 Enzymes, cofactors, supports, and other chemicals
Lyophilized alcohol dehydrogenase (EC 1.1.1.1) from baker’s yeast (YADH) (containing 60 % protein, 30 % sucrose, and 10 % phosphate) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The protein purity was 0.55 mg/mg of lyophilized powder with a specific activity of 330 IU/mg protein measured at 22 °C. Lyophilized LBADH (EC 1.1.1.2) was a recombinant Lactobacillus brevis alcohol dehydrogenase plain cell extract containing sucrose 5 times the protein amount (w/w), obtained from Juelich Fine Chemicals GmbH (Juelich, Germany). The protein purity was 0.17 mg/mg of lyophilized powder with a specific activity of 89 IU/mg protein measured at 30 °C. The second source of LBADH was the whole cells of a recombinant LBADH with a specific activity of 51 IU/mg of cell and obtained from Juelich Fine Chemicals GmbH (Juelich, Germany). Lyophilized ADH T was a recombinant Thermoanaerobacter species alcohol dehydrogenase heat-treated cell extract containing sucrose 5 times the protein amount (w/w), obtained from Juelich Fine Chemicals GmbH (Juelich, Germany). The protein purity was 0.13 mg/mg of lyophilized powder with a specific activity of 86 IU/mg protein measured at 30 °C. The second source of ADH T was the whole cells of a recombinant ADH T with a specific activity of 3.3 IU/mg of cell was and obtained from Juelich Fine Chemicals GmbH (Juelich, Germany).

\( \beta \)-nicotinamide adenine dinucleotide; oxidized form (\( \beta \)-NAD\(^+\)), \( \beta \)-nicotinamide adenine dinucleotide; reduced form (\( \beta \)-NADH), \( \beta \)-nicotinamide adenine dinucleotide phosphate; reduced form (\( \beta \)-NADPH), and \( \beta \)-nicotinamide adenine dinucleotide phosphate; oxidized form (\( \beta \)-NADP\(^+\)) were purchased from Biomol GmbH (Hamburg, Germany).

Non-porous plain glass beads (0.25 mm – 0.30 mm) were supplied by B. Braun Biotech International GmbH (Melsungen, Germany). Non-porous acid washed and unwashed glass beads (0.150 mm – 0.212 mm and 0.450 mm – 0.600 mm) were bought from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Non-porous Celite 545 (0.01 mm – 0.02 mm) and non-porous Celite 503 (<0.1 mm) were obtained from Merck KGaA GmbH (Darmstadt, Germany). Non-porous Polyamide particles (0.05 mm – 0.16 mm) was supplied by Carl Roth GmbH & Co. (Karlsruhe, Germany). Accurel (<1.5 mm) was purchased from Membrane GmbH (Obernburg, Germany). Silica gel 25 Å° (0.07 mm –
Chapter 3  Materials, Methods, and Experimental Set-up

0.14 mm) and Silica gel 60 A° (0.040 mm – 0.063 mm) were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ethanol, acetophenone, magnesium chloride (MgCl₂), trehalose, manganese chloride (MnCl₂), and tris-(hydroxymethyl)aminomethane hydrochloride (Tris) were bought from Merck KGaA GmbH (Darmstadt, Germany). Triethanolamine-hydrochloride (Tea), potassium dihydrogen phosphate (PO₄), and 2-(N-morpholino)ethane sulfonic acid (Mes) were purchased from Fluka Biochemika GmbH (Steinheim, Germany). Bradford reagent and lyophilized powder of bovine serum albumin (BSA) were supplied by Bio-Rad Laboratories GmbH (Munich, Germany), while silica gel orange, acetone, 2-Propanol, acetic acid, toluene, 3-(N-morpholino)propane sulfonic acid (Mops), potassium chloride, sodium chloride, potassium iodide, sodium hydroxide, and ammonium sulfate were bought from Carl Roth GmbH & Co. (Karlsruhe, Germany). 3-glycidoxypropyltrimethoxysilane, dichlorodimethylsilane, and sucrose were supplied by Sigma-Aldrich Chemie (Steinheim, Germany). Hexanal and hexanol were obtained from Lancaster Synthesis (England, UK).

3.2  Methods

3.2.1  Preparation of cell extract
Plain cell extract of LBADH or ADH T was prepared by sonicating a 20 % (w/v) cell suspension of the respective whole cells in a specific buffer solution for 5 min with 1 min break to dissipate the generated heat. A sonicator from Bandelin Electronics, Berlin, Germany was used for this purpose. After centrifugation at 5 °C for 20 min at $14 \times 10^3 \text{ g}$, the supernatant of the plain cell extract was collected. The heat-treated cell extract of ADH T was prepared by subjecting the plain cell extract of ADH T to heat treatment at 70 °C for 7.5 min (According to the supplier, ADH T enzyme was stable at 70 °C.). Then the extract was centrifuged at 5 °C for 20 min at $14 \times 10^3 \text{ g}$. The supernatant of the heat-treated cell extract was collected.

3.2.2  Pre-treatment of supports
The non-porous plain glass beads were washed with distilled water, and then dried.
In the second method, the non-porous plain glass beads were treated with 65 % (w/v) nitric acid. The resulting mixture was boiled for 5 min. Subsequently, the beads were washed with distilled water and dried at 105 °C for 24 h. The resulting non-porous plain glass beads were called acid-treated glass beads.

Hydrophilic glass beads were prepared by incubating acid-treated glass beads with 2 % (w/w) 3-glycidoxypropyltrimethoxysilane in toluene at room temperature for two weeks and then washed with distilled water. Then, they were treated with 10 % (w/w) acetic acid in order to open the epoxy group formed on the surface of the non-porous glass beads. The epoxy groups were formed by the reaction of the hydroxyl groups on the surface of the glass beads with 3-glycidoxypropyltrimethoxysilane. The mixture was heated in a water bath at 50 °C for 5 h. After removing the supernatant, the beads were washed with distilled water and dried at room temperature.

Hydrophobic glass beads were prepared by incubating acid-treated glass beads with 5 % (w/w) of dichlorodimethylsilane in toluene at room temperature. Then the mixture was shaken for 5 min and the supernatant was removed. The resulting beads were washed with distilled water and dried at room temperature.

Celites, Polyamide, Accurel, Silica gel 25 A°, and Silica gel 60 A° were pre-wetted with 50 ml ethanol for 30 min and then washed with 50 % (v/v) ethanol-water solution. Finally, the mixture was washed several times with distilled water and the wet beads were used for the immobilization process.

### 3.2.3 Immobilization of enzyme by physical adsorption

Unless otherwise stated, 1 ml of 100 mM PO₄⁻-buffer (pH 7.84) containing 4 mg protein of dissolved YADH was added to a 25 ml beaker containing 500 mg of support at 4 °C. The resulting mixture was stirred for 2 h with a magnetic stirrer (20 mm length and 6 mm diameter) at (125 ± 5) rpm. Then the beaker containing the whole enzyme/support suspension and the magnetic stirrer was placed in a desiccator containing dry silica gel orange, and the suspension was dried at 4 °C under an absolute pressure of 15 kPa, unless otherwise specified, until all water was removed from the suspension (see Figure 3.1). In this manner free flowing support particles were obtained. The time required for drying was recorded. The dried immobilized enzyme preparation was removed from the
beaker and finally stored at 4 °C. (It should be noted that part of enzyme remained in the beaker depending on the adsorptive affinity of the enzyme to the support material.) Similarly, the immobilization of LBADH was done using 50 mM Tea-buffer (pH 7) containing 1 mM MgCl₂, 2 mg of protein, and 500 mg of support. For immobilization of lyophilized ADH T, 50 mM Tris-buffer (pH 7), 2 mg of protein, and 500 mg of support were used. Immobilization of ADH enzymes from different sources were performed in the mentioned buffers at the mentioned concentrations because those buffers were used by the supplier for the enzyme preparation as standard. Immobilization of plain cell extract of LBADH and plain and heat-treated cell extract of ADH T was done using 170 units of the enzyme in a specific buffer and 500 mg of support.

![Figure 3.1: Schematic diagram of enzyme immobilization process](image)

**3.2.4 Determination of protein**

Bradford method (Bradford, 1976) was used to measure the protein concentration of solutions. 100 µl of enzyme solution was added to 900 µl of Bradford reagent in a 1 ml cuvette. Bradford reagent was prepared by diluting one volume of the reagent with four volume of distilled water. Then the resultant solution was filtered using filter paper from Schleicher and Schuell, Germany, having pore diameter of 4 µm to 7 µm. The solution was left for 5 min to allow the binding of the dye (Coomassie Brilliant Blue G-250) to
the protein. Thus, the reddish brown color of the dye was converted to blue color due to the binding of the dye by the protein. Then the absorbance was measured at 595 nm with a UVI KON 922 spectrophotometer (Kontron Instrument, Milano, Italy). Bovine serum albumin (BSA) was used as a standard protein. The calibration curve was obtained with known protein concentration of BSA by reading the absorbance of the diluted BSA at 595 nm. The absorbance versus protein concentration curve was linear in the restricted protein concentration range (between 0.01 mg and 0.07 mg protein/ml, corresponding to absorbance values between 0.1 and 0.6). Using the calibration curve, the protein concentration of the unknown protein solution was determined. All the measurements were done in triplicate and the average value was calculated and used.

The amount of protein loaded onto the dried support was determined according to the method described under so-called “465 Bradford assay” by Bonde, et al. (1992). This assay is basically a modified Bradford method and it measures the decrease in the absorbance of the solution at 465 nm due to adsorption of the dye by the bound protein. For this assay, 200 µl of distilled water was added to 4.5 mg of enzyme loaded support and 4.5 mg of blank support, respectively. 1800 µl of Bradford reagent, diluted (1:5) with distilled water, was subsequently added to each of these mixtures. After short agitation of the mixture (3 min) to allow binding of the dye with the protein, the mixtures were centrifuged at 18×10³ g. The absorbance of the two supernatants was measured at 465 nm, and this reading was then subtracted from the similar reading obtained with the blank support. The calibration curve for quantification of the protein was obtained with BSA as a reference protein at 465 nm. The protein loading on the dried support was calculated in terms of the ratio of mg of protein loaded on the support to the total mg of support taken.

### 3.2.5 Determination of enzyme activity

The activity of lyophilized YADH enzyme (before immobilization) was determined at 22 °C by adding 20 µl of a 12.5 mM β-NAD⁺ cofactor solution to 970 µl of 100 mM PO₄-buffer (pH 7.84) containing 100 mM ethanol as substrate. To initiate the reaction, 10 µl of the enzyme solution was added to the mixture. The increase in absorption at 340 nm due to formation of NADH was measured with the spectrophotometer. A molar extinction coefficient for reduced cofactor of 6.22 ml·µmol⁻¹·cm⁻¹ was used for
calculation of enzyme activity. One international unit [IU] of ADH enzyme was defined as the amount of enzyme required to convert (reduce or oxidize) one μmol of cofactor per min under the experimental conditions. By using this method, the activity of YADH before immobilization was found to be 330 IU/mg protein. The activities of the LBADH and ADH T were determined in a similar manner. For the assay of LBADH activity, 9.5 mM of β-NADPH in 50 mM Tea-buffer (pH 7) containing 1 mM MgCl₂, 11 mM acetophenone at 30 °C was used. For ADH T, 10 mM of β-NADPH containing 50 mM Tris-buffer (pH 7) with 10 mM of acetone for ADH T at 30 °C was used. The activities of the lyophilized LBADH and lyophilized ADH T were found as 89 IU/mg protein and 86 IU/mg protein, respectively. In a similar manner, the residual activity of the immobilized ADH enzymes was measured in aqueous solution by redissolving 3 mg of enzyme immobilized support in 100 ml buffer with their respective substrates and cofactor, as described for the activity determination of the ADH enzymes. The reaction mixture was stirred at 350 rpm for 10 min. 500 μl samples were periodically withdrawn and added into 500 μl ethanol in order to inactivate the enzyme. In a preliminary experiment, it was observed that all the three enzymes were inactivated by the resulting concentration of ethanol. The samples were then analyzed by measuring the change in absorption of the reduced nicotinamide cofactor at 340 nm. The enzyme activity was calculated according to the formula:

$$\text{Enzyme activity} = \frac{\Delta APM \times TV \times DF}{PL \times \varepsilon \times \text{reduced cofactor} \times SV}$$

(3.1)

where

- Enzyme activity is expressed in the international unit (IU)
- ΔAPM is the difference between the absorbance per minute of the sample and the absorbance per minute of the blank,
- TV is the total volume,
- DF is the dilution factor,
- PL is the path length,
- SV is the sample volume, and
- \(\varepsilon\) reduced cofactor indicates the extinction coefficient of the reduced cofactor measured at 340 nm and it is equal to 6.22 ml·μmol⁻¹·cm⁻¹.
Then the specific enzyme activity was defined as the ratio of enzyme activity [IU/ml] to the mg of protein per ml of the specific buffer solution [mg/ml]. Therefore,

\[
\text{Specific enzyme activity} = \frac{\text{Enzyme activity}}{\text{mg of protein}}
\]  \hspace{1cm} (3.2)

The activity of the immobilized enzyme was determined from the initial linear portion of the activity-time profile. Finally, the residual activity of ADH enzymes was calculated as a ratio of the specific enzyme activity measured after immobilization [IU/mg protein] to the specific enzyme activity before the immobilization [IU/mg protein] and was expressed as percentage.

3.2.6 Determination of enzyme stability

3.2.6.1 Aqueous phase
The thermo-stability of the ADH enzyme in aqueous phase was obtained by subjecting enzyme solution to a specific temperature in a thermo-constant bath. Samples were taken periodically and the residual activity of the enzyme was measured spectrophotometrically as described in Section 3.2.5. For this assay, 0.5 mg protein/ml containing NADP\(^+\) to protein ratio 0.01 (w/w) in a specific buffer solution was taken. The thermo-stability study of LBADH was carried out with 50 mM Tea-buffer (pH 7) containing 1 mM MgCl\(_2\) and ADH T in 50 mM Tris-buffer (pH 7), as the stability of these enzymes was reported to be high in the above mentioned buffer solutions by the supplier.

3.2.6.2 Gas-phase
The aim of the stability study was to screen among the various immobilization parameters based on the thermo-stability of the immobilized enzyme preparation at a specific temperature and relative humidity (RH) conditions, simulating the conditions of the gas-phase reaction system without substrate addition.

The standard procedure was to place a pre-weighed amount of the immobilized enzyme preparation into 1.5 ml Eppendorf tubes in a glass bottle containing 25 ml of a saturated salt solution. The saturated salt solution was used in order to reach the definite RH
inside the closed bottle at a specific temperature as depicted in Table 3.1. A filter paper was dipped in the saturated solution to increase the surface in order to obtain phase equilibrium of the system. Eppendorf tubes were placed on an aluminum foil placed on the saturated salt solution in a glass bottle, as shown in Figure 3.2. Samples were withdrawn periodically and the residual activity of the immobilized enzyme preparation was measured spectrophotometrically.

![Figure 3.2: Sketch of experimental set-up for measurement of thermo-stability of enzymes in gas-phase](image)

<table>
<thead>
<tr>
<th>Temperature [°C]</th>
<th>Saturated potassium iodide [%]</th>
<th>Saturated sodium chloride [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 °C</td>
<td>67.89 ± 0.23</td>
<td>75.09 ± 0.11</td>
</tr>
<tr>
<td>60 °C</td>
<td>63.11 ± 0.31</td>
<td>74.50 ± 0.30</td>
</tr>
</tbody>
</table>

3.2.7 Scanning electron microscopy

In order to gain information about the distribution of protein on the glass bead surface, the surface of the protein loaded glass beads was photographed using a scanning electron microscope (JSM 6400, Jeol, Peabody, USA). Glass beads were fixed on the aluminium plate of 2 cm with carbon tape. Then the samples were coated with a thin platinum layer under argon atmosphere to ensure the electron conductivity and analyzed under high vacuum with a tungsten capillary cathode. This was done at Institute for Ceramic Components under Faculty of Mechanical Engineering of RWTH Aachen.
3.2.8 Reaction system

In this study YADH, LBADH, and ADH T were employed to catalyze the reduction of carbonyl compounds to their respective alcohols. The stoichiometrically consumed cofactor was regenerated using a short chain alcohol, which was oxidized by the same enzyme.

YADH catalyzed the reduction of hexanal to 1-hexanol. The stoichiometrically consumed cofactor was regenerated using ethanol as a cosubstrate, which was oxidized by the same enzyme (Figure 3.3).

![Figure 3.3: Scheme for standard reaction with YADH](image)

Reduction of acetophenone to (R)-phenylethanol and (S)-phenylethanol was catalyzed by LBADH and ADH T, respectively, (Figure 3.4). The stoichiometrically consumed cofactor was regenerated with the same enzyme using isopropanol as a cosubstrate.

![Figure 3.4: Scheme for standard reaction with LBADH and ADH T](image)

3.2.9 Batch gas-phase reaction

The batch gas-phase reactions were carried out using batch reactors. One such batch reactor is shown in Figure 3.5. Details of the drawing are shown in Section 3.3.1 and in
Appendix A. The standard reaction procedure consisted of the following steps. At first, a definite amount of a specific saturated salt solution containing two substrates (a main substrate and a coupled substrate for cofactor regeneration) was poured into a glass bottle. The saturated salt solution was used to maintain a specific RH and it served as a reservoir for substrates. Then the weighed amount of immobilized enzyme preparation was placed onto the membrane filter paper from Schleicher and Schuell, Germany, having pore diameter 1 µm in the reactor (in the head space portion of the reactor). After that the reactor was tightly closed and placed onto a magnetic stirring plate in a thermo-constant chamber at a specific temperature. The point of the beginning of the stirring action was defined as the start of the reaction. Samples were taken at definite intervals from the liquid-phase with a plastic one-way syringe (B. Braun, Melsungen, Germany) and from the gas-phase with gas tight syringe (Hamilton, Reno, USA). The samples were then further processed as described in Section 3.2.11.1 and Section 3.2.11.2. All batch experiments were performed in duplicates, which were analogously prepared and ran in parallel.

Figure 3.5: Batch gas-phase reaction using reactors each placed separately inside each glass bottle containing saturated salt solution, substrates, and immobilized enzyme preparation stirred on magnetic stirring plate

3.2.10 Continuous gas-phase reaction

The sketch of the continuous gas-phase reactor is shown in Section 3.3.2. The thermo-constant chambers were first set to a desired reaction temperature. Using dried nitrogen as a carrier gas, the flow of the substrates were regulated using the flow controllers. The gas was flowed through the reactor by-passing the bioreactor till the equilibrium of the
system was attained. A definite amount of immobilized enzyme preparation was plugged inbetween the dry glass wool in the bioreactor. The reaction was started by allowing the substrate flow through the bioreactor. The online chromatography was programmed and automatic sampling was started. The samples were analyzed by the online gas chromatography as described in Section 3.2.11.4.

3.2.11 Gas chromatography

3.2.11.1 Preparation of liquid samples
In the batch gas-phase reactor, the presence of the saturated salt solution and isopropanol (which was present as co-substrate for cofactor regeneration) enhanced the solubility of the substrate and rendered the extraction process more difficult. Ethyl acetate was used as a standard extraction solvent because of its good extraction capabilities and low toxicity (Ferloni, 2004). As per the standard procedure, 250 µl of liquid-phase was sampled and extracted with an equal amount of ethyl acetate. 200 µl of organic phase (from the extract of ethyl acetate) was added to the gas tight vial and analyzed by gas chromatography (GC), as mentioned in Section 3.2.11.3.

3.2.11.2 Preparation of gas samples
250 µl of the gas samples were taken with a gas tight syringe from the batch reactor at regular intervals. The gas sample was dissolved in the 150 µl cold ethyl acetate solution containing 0.01 mM decane as internal standard in a gas tight vial and the samples were analyzed by gas chromatography (GC), as described in Section 3.2.11.3.

3.2.11.3 Off-line analysis
The gas and the liquid samples from the batch reactor were analyzed using a Varian gas chromatograph CP-3800 (USA) equipped with an autosampler (CP 8400, Varian Inc., USA), a flame ionization detector and a column CP-WAX 58 (FFAP) CB (25 m x 0.25 mm x 0.2 µm; Chrompack Inc., USA). The temperature of the injector and the detector was kept at 210 °C and 280 °C, respectively. The injector split (dilution of the sample with nitrogen in a specific ratio) as well as the column oven temperature were optimized for each compound to avoid peak broadening due to the presence of high concentration of compounds and to separate the peaks well. Nitrogen was used as carrier gas with a constant flow of 0.5 ml/min. All samples were analyzed in triplicate. The results were
analyzed with the Star Workstation 5.5 software (Varian Inc., USA) using a five-point calibration, acquired each time directly before a new series of samples. The conditions of the gas chromatography (GC) for each analyzed compound are reported in Table 3.2.

Table 3.2: Gas chromatography program for the analysis of substances

<table>
<thead>
<tr>
<th>Substances</th>
<th>Split</th>
<th>Column oven program</th>
<th>Total time of the program [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetophenone/phenylethanol</td>
<td>1:20</td>
<td>40 °C for 6 min, 20 °C/min till 180 °C, 180 °C for 4 min</td>
<td>17</td>
</tr>
<tr>
<td>Hexanal/hexanol</td>
<td>1:20</td>
<td>45 °C for 10 min, 25 °C/min till 180 °C, 180 °C for 1 min</td>
<td>16.4</td>
</tr>
</tbody>
</table>

3.2.11.4 On-line analysis

The outlet of the continuous gas-phase reactor was connected directly to a six-way valve provided with a 250 µl loop, as described by Ferloni, (2004). The switching of the valve and the gas chromatography (GC) itself were controlled through the HPGC-Chemstation software Rev. A.07.01, with which the results were evaluated. The connection between gas-phase reactor and GC was kept at a temperature at least 10 °C higher than the operational temperature of the reactor, to avoid condensation, while the six way valve was kept at 100 °C temperature. At definite intervals, 250 µl of the gas coming out at the reactor outlet was automatically injected in a HP 5890 GC equipped with a split-splitless injector. The split by the injection was kept at a constant value of 1:20. The injector and the detector temperatures were 170 °C and 250 °C, respectively. The GC was equipped with a fused silica column CP-WAX 57 CB (25 m×0.32 mm×0.2 µm; Chromopack Inc., USA) and a flame ionization detector. Nitrogen was used as the carrier gas with a head pressure of 120 kPa for the GC. The temperature program for the detection of isopropanol, acetophenone, and phenylethanol was of total 18.6 min. The initial temperature of the GC column in the oven was maintained at 40 °C for 5 min and then a ramp of 15 °C/min till 180 °C was continued with 5 min hold at 180 °C.
3.3 Experimental set-up

3.3.1 Batch gas-phase reactor

The initial concept of batch reactor for enzymatic gas-phase catalysis was developed from the previous studies of Ferloni, (2004). The aim was to have a batch reactor in large numbers so as to conduct large number of parallel experiments in short duration. (The time taken for each experiment in a continuous reactor was usually very long.)

![Diagram of batch gas-phase reactor](image1)

Figure 3.6: Drawing of the first version of batch gas-phase reactor (A) and complete set-up of the first version of batch gas-phase reactor (B)

The drawing of the first version of the batch reactor used in this study is shown in Figure 3.6A. The immobilized enzyme preparation was placed onto a membrane filter paper from Schleicher and Schuell, Germany, having pore diameter 1 µm that was fixed between two Teflon rings in a metal basket. The reactor was placed into a glass bottle containing a defined substrate concentration in a specific amount of a saturated salt solution. The saturated salt solution maintained a specific RH in the gaseous environment of the closed system at a specific temperature (Figure 3.7B). The whole reactor was placed onto a magnetic stirring plate and stirred at a specific speed. The equilibrium of the substrates between the liquid and the gas-phase was established in a closed system within short duration. Only the molecules in the gas-phase reacted with the immobilized enzyme preparation and were converted, while the liquid-phase acted...
as a reservoir for substrates and products. As soon as the gaseous substrate molecules reacted with the enzyme, the phase equilibrium of the substrates between the gas and the liquid-phase favored the transformation of the new substrate molecules from the liquid to gas-phase, at the same time the gas-phase molecules were condensed in the liquid-phase. The upper and lower end of the basket shaped metal was open so that the gas could diffuse through the membrane filter paper. The two openings on the wall of the glass bottle closed with silicon septum were used for sampling from the liquid and the gas-phase. During the experiments, it was observed that the performance of the reactor was highly dependent on the magnetic stirrer that was used for stirring the solution. Also the position of the magnetic stirrer was not constant, and often the stirrer was stuck and lost the synchronization with the magnetic stirring plate. All the above factors revealed the necessity for modification of the batch reactor.

The first modification was termed as hollow shaft batch reactor. The final design of this reactor is shown in Figures 3.7. The idea behind the construction of the hollow shaft batch was that the high-speed rotating stirring disc would generate a convective gas flow for the movement of the gas-phase and at the same time would mix the solution. A specially designed stirring disc contained a fixed single row of strong magnets to create sufficient magnetic power for rotation at high speed. The stirring disc had two holes in a straight line connecting to each other and was connected directly to the hollow shaft to let out the sucked gas into the solution. The stator and the stirring disc were made of PEEK (polyetheretherketone) material and the hollow shaft was made from stainless steel (V4A). The stator itself was made with two pieces of PEEK, one embedded inside the other. The first piece formed the outer portion of the stator and the second piece formed the inner portion of the stator. The hollow shaft passed through the hole present in the center portion of the second piece of PEEK. The diameter of this hole was greater at the middle portion as compared to the ends in order to reduce the friction between the inner walls of the stator and the outer metal surface of the hollow shaft. This second piece of PEEK held the hollow shaft in the stator to minimize the lateral vibration motion of the hollow shaft generated at a high stirring speed of the stirring disc. In the preliminary study, it was found that sufficient power to stir the solution was not achieved even at a very high speed. This was because of the thickness of the stirring disc, which in turn increased the distance between the magnetic stirring plate and the stirring disc of the reactor. Moreover, due to the formation of the vortex the further flow
of the gas was hindered. To this end, baffles (of specific dimensions) were attached to the stator. However, the gas from outside was taken up from edges of the baffles, which hindered the flow of the gas through the hollow shaft. A small amount of gas was sucked in the small gap between the stator and the hollow shaft. Unfortunately, the gas bubbles coming from the edge of the baffles settled at the base of the stator near the small gap between the hollow shaft and stator and thus hindered the further flow of the gas coming from the small gap. Bubbles were also settled at the stirring metal tip and with time, these bubbles grew larger and larger till they stopped the stirring movement of the stirring disc. The overall convective flow of the gas in the reactor was very low. A small cut of 1 mm was made on the stirring disc at the front of the two holes on the stirring disc (slanting in left) to create eddies at the edge of the cut during the rotation of the stirring disc, which, in turn, might suck some gas through the hollow shaft. Still there was no improvement in the gas flow performance.

Figure 3.7: Drawing of the first modification of the batch gas-phase reactor (hollow shaft reactor)

The second modification was termed as modification 2. Trials were made to prevent the suction of the gas from the edges of the baffles (Figure 3.8). Number of changes in the earlier constructions was made in order to obtain high convective flow of the gas through the reactor column. In the final version of modification 2, the hollow shaft was
replaced with the stirring rod of stainless steel, the thickness of the stirring disc was reduced. The lower portion of the stator was angled. The stirring rod passed through the center hole present in the stator. In addition eight holes were created in stator passing from top to the bottom of the stator to enhance the flow of gas through these holes in the solution in small bubbles. The angle of the holes on the stator and stirring disc was adjusted so that the gas coming from the stator would pass through the stirring disc holes into solution instead of bubbles accumulating on the lower portion of the stator and the tip of the stirring rod. During the trials, it was found that a very minor amount of gas flew through the holes of the stator and the bubbles were coming from the edges of the baffles. Also it was observed that the distance between the stirring disc and stator influenced the size of bubbles coming from the edges of the baffles. Although at a specific distance, some gas was about to come from the stator holes, but the intensity of the gas bubbles coming from the edges of the baffles was so high that the gas was not able to come from the stator holes. The phenomenon described in the hollow shaft reactor reappeared in this case also. In addition, since the stator was made from single piece of PEEK that held the stirring rod, friction was generated between the outer surface of the metal stirring rod and the inner wall of the stator and the stirring disc vibrated at high stirring speed.

![Diagram of the second modification batch gas-phase reactor](image)

**Figure 3.8:** Drawing of the second modification batch gas-phase reactor
The third modification was termed as Frings turbine 1st. The basic idea of the construction of this batch reactor was based on the concept of rotor-stator principle developed by the company Frings GmbH (Bonn, Germany). The self-gassing turbine from Frings were composed of a hollow rotor rotating at a high velocity inside a specially designed stator. During the rotation, the gas within the rotor was sucked into the system through the openings at the end of the rotor wings. This concept, usually employed for the simultaneous mixing and gassing of the fermentation broth, was slightly modified for the construction of the enzymatic gas-phase batch reactor system. As shown in the final design of Frings turbine 1st in Figure 3.9, the stator made from stainless steel (V4A) was a single piece with a specific angled ten tooth in a clockwise direction (see Appendix A). The stator was made up of specific construction so as to fit the rotor with the stirring rod (made from stainless steel V4A). The six angled wings of the rotor (clockwise direction) were very close to the ten angled tooth of the stator (clockwise direction) with a very small gap between them. The stator contained four holes of larger diameter (4 mm) for the flow of gas and one hole of smaller diameter (3.2 mm) in the center to fix the stirring rod. The upper portion of the stator was not flat, but angled. The rotor and the stirring disc containing a single row of strong magnets, were made from a single piece of PEEK and the stirring metal rod was attached in the center. The distance between the rotor wings and the stirring disc was 7.8 mm. The tip of the stirring rod was pressed sufficiently so as to minimize the distance between the stirring disc and the magnetic stirring plate. In a preliminary study, it was found that the performance of this modification was better compared to the modifications 1 and 2. The stirring of the rotor wings created vacuum in the small gap between the rotor wings and the stator tooth. The gas was sucked from the upper surface of the stator through the holes present in the stator and then it was thrown into the solution in bubbles. These bubbles rose to the surface of the solution and then the gas was freed in the reactor. It was observed that the increase in stirring speed increased the intensity of the vacuum in the small gap between the stator and rotor and enhanced the convective gas flow. The lower the level of the solution the lesser was the resistance for the movement of rotor and therefore the gas flow was better. The following points were observed during the operation of the Frings turbine 1st. At higher than 1200 rpm stirring speeds, the stirrer vibrated indicating that the magnetic strength of the stirring disc was not sufficient. Since the stator was made from single piece of stainless steel, therefore friction was generated between the outer surface of metal stirring rod and the inner wall of the stator.
that hindered the gas flow. Moreover, the upper portion of the stirring rod was not fixed and it created difficulties in placing the reactor in the glass bottle. It was noticed that the rotor wings and the stator tooth were angled in a direction opposite to the specifications of Frings company Appendix A). In order to overcome the aforesaid problems and correct the reactor construction, further modifications were made in Frings turbine I\textsuperscript{st}.

![Figure 3.9: Drawing of the third modification batch gas-phase reactor (Frings turbine I\textsuperscript{st})](image)

The fourth modification was termed as Frings turbine II\textsuperscript{nd}. The final design is shown in Figure 3.10. (The detail drawing of each part of Frings turbine II\textsuperscript{nd} can be found in Appendix D.) The stirring disc was equipped with two rows of the magnets to develop sufficient magnetic power for stirring at higher speeds. The tip of the stirring rod was pressed sufficiently in the stirring disc to minimize the distance between the stirring disc and the magnetic stirring plate. The distance between the stirring disc and the rotor wings was reduced to 7 mm and also the height of the upper portion of the rotor (above the rotor wings) fitting the rotor in the stator was reduced. The stator portion was made from two pieces of PEEK, one piece fitting inside the other. First piece formed outer portion of the stator and the second piece formed the inner portion of the stator. This second piece of PEEK had four holes for the gas flow and one hole in the center to fix the stirring rod. The diameter of this center hole was greater in the middle portion to reduce the friction between the outer surface of metal stirring rod and inner surface of
the stator. The ends of this center hole were of smaller diameter to hold the stirring rod and to prevent the lateral vibration of the stirring metal rod at high stirring speed. A small screw was placed at some distance above the stator to hold the upper portion of the stirring rod and to prevent upward and downward vibration of the stirring rod at higher stirring speed. Care was taken to prevent the friction between the screw and the upper surface of the stator. The upper portion of the stator was made flat and two hollow rings of POM (polyoxymethylene) material were placed onto it. The first POM ring was served as a base to hold the membrane filter paper on which the second POM ring was served as a column for enzyme loading. The inner diameter of the hole of second POM ring was equivalent to the inner diameter of the glass column used for enzyme loading in the continuous reactor. The length of the stirring rod was defined so as not to interfere with the placing of the membrane filter paper. The direction of the wings of the rotor was corrected to anticlockwise. Surprisingly, the correct direction (anticlockwise) of the angled stator tooth in the actual Frings turbine did not result in good performance, therefore, they were angled in clockwise direction.

![Diagram of the fourth modification batch gas-phase reactor](image)

Figure 3.10: Drawing of the fourth modification batch gas-phase reactor (Frings turbine II\textsuperscript{nd})
3.3.2 Continuous gas-phase reactor

Figure 3.11: Sketch of continuous gas-phase reactor

The continuous gas-phase reactor was composed of two thermo-constant chambers equipped with the substrate vaporization unit and the reaction unit. They were connected to each other by a connector maintained at a constant temperature using a thermo-constant water bath (Figure 3.11). The substrate vaporization unit consisted of substrate flasks (main substrate, co-substrate and water). The reaction unit containing the bioreactor was composed of a glass tube (20 cm length and 6 mm inner diameter) in which a defined amount of immobilized enzyme preparation was packed inbetween dry glass wool layers. Nitrogen was used as a carrier gas. The gas was dried over silica gel and molecular sieves prior to being saturated with substrate and water. Substrate feeding was done by passing dried nitrogen as a carrier gas through the substrate flasks. Substrate saturated nitrogen from different lines were mixed and then continuously flown through the bioreactor, reacting with the immobilized enzyme preparation. The desired thermodynamic activities of each compound (see Section 2.7) were obtained by
adjusting the volumetric flows of the carrier gas in the different lines at appropriate values using a flow controller. The gas leaving the bioreactor was injected at definite intervals into an on-line gas chromatography (see Section 3.2.11.4) for analysis and the remaining gas was condensed in a cold trap.
4 RESULTS AND DISCUSSION
4.1 Optimization of enzyme immobilization parameters

This study was attempted to investigate the effect of various immobilization parameters on the immobilization efficiency of ADH enzymes. The parameters were stirring temperature, stirring time, stirring speed, drying temperature, drying pressure, drying time, addition of different stabilizers (sucrose, buffers, etc.), amount of added protein, and supports. The immobilization efficiency was judged in terms of the residual activity and the protein loading.

4.1.1 Effect of temperatures on immobilization efficiency

![Figure 4.1: Effect of temperatures on immobilization efficiency of lyophilized YADH: Black bars represent residual activity and white bars denote protein loading. Immobilization conditions: 4 mg of lyophilized YADH, 500 mg of non-porous plain glass beads, 1 ml of 100 mM PO₄-buffer (pH 7.84), 2 h stirring time, (125 ± 5) rpm stirring speed, 15 kPa absolute drying pressure.](image)

Three combinations of stirring and drying temperatures for immobilization of YADH were chosen and their effect on the immobilization efficiency was observed. This is shown in Figure 4.1. Low residual activity (30 %) was obtained when both stirring and drying were done at high temperature (22 °C), while stirring at low temperature (4 °C) and drying at high temperature (22 °C) resulted in higher residual activity (48 %). This activity was further increased to 60 %, when the stirring and drying was carried out at a
low temperature (4 °C). Thus, low temperature was found to be less detrimental to the enzyme during stirring and drying. This observation is in line with the findings of Liao and Chen, (2001), who explained this behavior due to reduced thermo-inactivation of the enzyme at lower temperature. However, Figure 4.1 does not reveal a notable change in the protein loading on the non-porous plain glass beads with different stirring and drying temperatures. Only 37.5 % of the initially added protein was adsorbed on the non-porous plain glass beads.

### 4.1.2 Effect of stirring speed and stirring time on immobilization efficiency

![Graph A: Effect of stirring speed on residual activity and protein loading](image)

![Graph B: Effect of stirring time on residual activity and protein loading](image)

Figure 4.2: Effect of stirring speed (A) and stirring time (B) on immobilization efficiency of lyophilized YADH: (■) represents residual activity and (□) denotes protein
loading. Immobilization conditions: 4 mg of lyophilized YADH, 500 mg of non-porous plain glass beads, 1 ml of 100 mM \(\text{PO}_4\)-buffer (pH 7.84), 4 °C temperature, 15 kPa absolute drying pressure, (A) 2 h stirring time, (B) (125 ± 5) rpm stirring speed.

In a preliminary experiment, it was found that the unstirred sample had a non-uniform protein loading on the non-porous plain glass beads. Therefore, the immobilization of YADH on the glass beads was performed at four different stirring speeds (50 rpm, 150 rpm, 250 rpm, and 400 rpm) keeping the adsorption time (2 h) constant. The results are presented in Figure 4.2A. Evidently, the residual activity decreased with increasing stirring speed. This is attributed to detrimental shearing effects on the enzyme caused by the magnetic stirrer, as previously reported by several researchers (Kaya, et al. 1994 and Charm and Wong, 1981). Prolonged exposure of the enzyme to stirring reduced its activity, as shown in Figure 4.2B. This is also due to the shear forces induced by the stirrer, as reported by Kaya, et al. (1994) and Charm and Wong, (1981). Figure 4.2 tells that stirring speed and time had no influence on the protein loading of the non-porous plain glass beads.

### 4.1.3 Effect of drying conditions on immobilization efficiency

Dehydration is known as stress to proteins potentially causing protein unfolding (Depaz, et al. 2002). Hence, a detailed investigation of the drying process and its effects on the residual activity as well as on the protein loading was conducted. Drying of the enzyme/glass bead suspension was performed at seven different absolute pressures starting from the atmospheric pressure (100 kPa) down to 4 kPa. The results are shown in Figure 4.3. The time required for drying of the preparation was significantly reduced with a decrease in absolute pressure. This can be explained by the inverse relation between the diffusion coefficient and the pressure (Reid, et al. 1987), which resulted in enhanced mass transfer from the aqueous solution to the silica gel at low pressure (see Figure 3.1). Evidently, there was a minor decrease in protein loading with the increase in absolute pressure. A very interesting relation between the drying pressure and the residual activity was found. With decreasing absolute pressure from the atmospheric pressure, the residual activity started increasing till a pressure 45 kPa was reached, then it started decreasing. At the optimum pressure (45 kPa), the residual activity was found as 69 %. Interestingly, this value was nearly 7 % higher than the highest residual activity (62 %) reported till date in the literature for the immobilized YADH (Liao and...
Chen, 2001). A tentative explanation behind the low residual activity obtained at higher absolute pressure resulting in extended drying time can be obtained from Lui, et al. (1991). According to them, prolonged drying of some enzymes at high absolute pressure causes inter-molecular S-S bond formation via thiol-disulfide interchange reactions of cysteine residues, which results in loss in enzyme activity. However, low residual activity was also obtained at a lower pressure. This effect was very critically studied. By visual inspection of the enzyme/glass bead suspension during the drying process, it was found that air dissolved in the solution formed bubbles. This bubble nucleation could result in detrimental effect on enzyme activity. In order to confirm this, an experiment was conducted, where after the 2 h stirring of the enzyme/glass bead suspension, helium was pumped through the gaseous headspace of the closed bottle for 30 min in order to strip off the air from the solution. This stripping was achieved by the equilibration of the helium gas-phase and the water phase during the employed period of time. Since helium has a low solubility in water compared to oxygen and nitrogen (Lide, 1999), only a significantly reduced amount of dissolved gas (helium) was finally present in the aqueous solution. After this degassing step, the solution was subsequently dried at 4 kPa absolute pressure. As shown by the right-most black bar in Figure 4.4, the residual activity of 63 % was found as compared to the residual activity of 34 % obtained with the same pressure without preliminary degassing of the enzyme/non-porous plain glass beads suspension (first black bar from left in Figure 4.4). This gain in residual activity confirmed that the bubble nucleation was indeed responsible for the reduced residual activities at a low pressure.

In order to test whether more steps in the drying process could further minimize the bubble nucleation, the drying of the enzyme preparation was performed in several steps: the first step was at 70 kPa for 2 h, followed by 45 kPa for 2 h, then 15 kPa for 2 h, while the rest of the drying was continued at 4 kPa. This experiment resulted in 69 % residual activity, as shown by the right-most black bar in Figure 4.5. Almost the same residual activity was also obtained in one-step drying process at 45 kPa and two-step drying, as shown by the first and second black bar from left in Figure 4.5, respectively. Thus, the two step drying process was not only effective in retaining good residual activity but also significantly decreased the drying time as compared to the one-step and multiple-step drying processes.
Figure 4.3: Effect of drying pressure on immobilization efficiency of lyophilized YADH: (□) represents residual activity, (□) denotes protein loading, and (○) represent drying time. Immobilization conditions: 4 mg of lyophilized YADH, 500 mg of non-porous plain glass beads, 1 ml of 100 mM PO₄-buffer (pH 7.84), 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed. (The standard deviation of residual activity was approximately 1.5 %.)
Chapter 4  Results and Discussion

Figure 4.4: Effect of drying pressure on residual activity and drying time of lyophilized YADH: Black bars represent residual activity and white bars denote drying time. Immobilization conditions were the same as mentioned against Figure 4.3.

![Graph showing effect of drying pressure on residual activity and drying time of YADH]

Figure 4.5: Effect of drying conditions on residual activity of lyophilized YADH: Black bars represent residual activity. Immobilization conditions were the same as mentioned against Figure 4.3.

As with YADH, the immobilization of LBADH was also carried out under various drying pressures. The results are shown in Figure 4.6A. With LBADH, the maximum residual activity was also achieved at a drying pressure of 45 kPa reaching 316 %. Interestingly, the residual activity of LBADH at all pressures was markedly higher than that of the native enzyme at all the pressures. In this regard, with a view to the hyperactivation of lipases by selective interfacial adsorption, as mentioned by Bastida, et al. (1998), it was hypothesized that the adsorptive immobilization of LBADH enzyme might be leading to some structural changes in the enzyme molecule, which was retained even after redissolving the immobilized enzyme in aqueous solution during its activity determination. In the case of LBADH, there was a slight decrease in protein loading with the increase in drying pressure. At low pressure, about 40 % of the initially added protein was adsorbed on the non-porous plain glass beads in contrast to 35 % for YADH.
The residual activity behavior of ADH T at different drying pressures is depicted in Figure 4.6B. From the atmospheric pressure down to 15 kPa, a nearly 100% recovery of enzyme activity was observed. Only at the lowest pressure (4 kPa), a significant decrease of residual activity was found. The reason was most probably due to bubble nucleation at this condition. This indicates that thermophilic ADH T was more stable at drying pressures of 15 kPa – 45 kPa as compared to the mesophilic LBADH and YADH enzymes. Among the mesophilic alcohol dehydrogenase enzymes, YADH was found to be most sensitive to drying pressures. The protein loading behaviors of ADH T and LBADH were very similar. The theoretical protein loading (see Section 4.1.6) of these two ADH enzymes was 0.0028 mg per mg of non-porous plain glass beads. About 70% of each of these proteins relative to the theoretical protein loading was loaded on the support.
Figure 4.6: Effect of drying pressure on immobilization efficiency of lyophilized LBADH (A) and lyophilized ADH T (B): (□) represents residual activity and (□) denotes protein loading. Immobilization conditions: 2 mg of lyophilized LBADH or lyophilized ADH T, 500 mg of non-porous solid glass beads, 1 ml of 50 mM Tea-buffer (pH 7) containing MgCl₂ for LBADH, 1 ml of 50 mM Tris-buffer (pH 7) for ADH T, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed.

Figure 4.7: Effect of drying pressure on immobilization efficiency of lyophilized YADH using Silica gel 25A° as support: (□) represents residual activity and (□) denotes protein loading. Immobilization conditions: 4 mg of lyophilized YADH, 500 mg of support, 1 ml of 100 mM PO₄-buffer (pH 7.84), 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed.
Figure 4.8: Effect of drying pressure on immobilization efficiency of lyophilized YADH using Silica gel 60A° as support: (||) represents residual activity and (□) denotes protein loading. Immobilization conditions were the same as mentioned against Figure 4.7. The effect of pressure during drying on the immobilization efficiency of lyophilized YADH on other supports such as Silica gel 25 A° and Silica gel 60 A° was studied as shown in Figures 4.7 and 4.8. It was found that the behaviors of the residual activity and the protein loading of the immobilized YADH with the change in drying pressure followed a similar trend as those with non-porous plain glass beads.

### 4.1.4 Effect of additives on immobilization efficiency

#### 4.1.4.1 Sucrose and others

It is known that sucrose stabilizes the enzyme by forming an amorphous phase with the protein and hydrogen bonds in place of water (Carpenter, et al. 1989). For a better understanding of this effect, an experiment was conducted, where an amount of sucrose equal to 5 times of the protein amount (w/w) was added to the protein/glass bead suspension, before the drying process was performed at 45 kPa absolute pressure (Figure 4.9A). As a result, 79% residual activity was obtained. This was nearly 17% higher than the highest residual activity (62%) reported in the literature till date for immobilized YADH (Liao and Chen, 2001). Thus, the role of sucrose to protect protein during drying was significant. Similar effects were reported by Carpenter, et al. (1989). Protein loading was slightly reduced when sucrose was added. Obviously, sucrose occupied some adsorption sites on the support material and consequently fewer sites on the support were available for protein binding. The lyophilized LBADH already contained sucrose 5 times the protein amount (w/w), therefore, an addition of sucrose 5 times the protein amount (w/w) meant the presence of sucrose 10 times the protein amount (w/w) was studied. It was observed that addition of sucrose decreased the residual activity (Figure 4.9B). This observation is in line with the findings of Monsan and Combes, (1984), who explained that sucrose, at high concentration forms sucrose intra-molecular and sucrose-sucrose intermolecular hydrogen bonds instead of hydrogen bonding with the protein. This gives a folded and aggregated structure for sucrose
molecules that are not bound to enzyme, leading to loss of enzyme activity. The decrease in protein loading with the addition of sucrose was due to the same reason as mentioned for YADH. Similar to lyophilized LBADH, addition of sucrose 10 times the protein amount (w/w) was studied with lyophilized ADH T that already contained sucrose 5 times the protein amount (w/w). Like LBADH, the addition of sucrose 10 times the protein amount (w/w) resulted in decrease in residual activity of ADH T (Figure 4.9C) owing to the same reason as mentioned for LBADH. Also in this case, the protein loading decreased with addition of sucrose due to the same reason as described for YADH.

Figure 4.9: Effect of sucrose addition on immobilization efficiency of lyophilized YADH (A), lyophilized LBADH (B), and lyophilized ADH T (C): Black bars represent residual activity and white bars denote protein loading. Immobilization conditions: 4 mg of lyophilized YADH, 2 mg (corresponding to 170 units) of lyophilized LBADH or lyophilized ADH T, 500 mg of non-porous plain glass beads, 1 ml of 100 mM PO$_4^-$-buffer (pH 7.84) for YADH, 1 ml of Tea-buffer (pH 7) with 1 mM MgCl$_2$ for LBADH, 1 ml of Tris-buffer (pH 7) for ADH T, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure, with sucrose: sucrose added 5 X and 10 X (X stands for times) the protein amount (w/w).

In order to investigate the effect of sucrose amount on immobilization efficiency, the immobilization of plain and heat-treated cell extract (fairly purified) of ADH T was performed at different amounts of sucrose, as depicted in Figures 4.10A and B. It was
found that the residual activity increased with the increase in sucrose addition till 5 times the protein amount (w/w). Further increase in sucrose addition to 10 times the protein amount (w/w) decreased the residual activity. The protein loading decreased with the increase in sucrose addition owing to the same reason as mentioned earlier for YADH. Interestingly, the hyperactivation of ADH T enzyme was found with both plain and heat-treated immobilized enzyme preparations. It was hypothesized that similar to lyophilized LBADH adsorptive immobilization of plain and heat-treated cell extract of ADH T might have lead to structural changes in the enzyme molecule which was retained even after dissolving the enzyme in aqueous solution for activity determination.

Figure 4.10: Effect of sucrose addition on immobilization efficiency of plain cell extract of ADH T (A) and heat-treated cell extract of ADH T (B): Black bars represent residual activity and white bars denote protein loading. Immobilization conditions: 170 units of plain or heat-treated cell extract, 500 mg of non-porous plain glass beads, 1 ml of 50
mM PO₄-buffer (pH 7), 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure, with sucrose: sucrose added 1X, 2X, 5X, and 10X (X stands for times) the protein amount (w/w).

In general, it was found that the addition of sucrose up to 5 times the protein amount (w/w) increased the residual activity and further addition of sucrose decreased the residual activity. Surprisingly, the immobilized heat-treated cell extract (Figure 4.10B) showed hyperactivation in comparison to the immobilized lyophilized ADH T (Figure 4.9C), which was originally lyophilized from the heat-treated cell extract. This might be due to two reasons: inactivation of the enzyme due to lyophilization of heat-treated cell extract and/or the buffer used for immobilization. This was investigated and the results are discussed in Section 4.1.4.2.

The effect of other stabilizers such as BSA, metal salts (NaCl, KI, MgCl₂, and MnCl₂), and polyols (sucrose and trehalose) on the immobilization efficiency of lyophilized ADH T was also studied. The results are shown in Figure 4.11. It was found that the response of immobilized lyophilized ADH T was different to different stabilizers. Comparing Figure 4.11 with Figure 4.10, it can be said that the retained residual activity was less as compared to the immobilized plain and immobilized heat-treated cell extract preparations of ADH T.
Figure 4.11: Effect of stabilizers on immobilization efficiency of lyophilized ADH T: Black bars represent residual activity and white bars denote protein loading. Immobilization conditions: 2 mg of lyophilized ADH T, 500 mg of non-porous plain glass beads, 1 ml of 50 mM Tris-buffer (pH 7), 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure, with stabilizers: stabilizers 5X (X stands for times) the protein amount (w/w).

4.1.4.2 Buffers
The effect of buffer pH on the immobilization efficiency of plain cell extract of ADH T was carried out by varying the pH of the PO₄-buffer within its pKa range at a constant buffer ionic strength. It was found that the residual activity increases with the increase in pH from 6.5 to 8 (Figure 4.12). It can be clearly seen that an optimum residual activity of the immobilized enzyme was obtained at pH 8. This activity is higher than the optimum activity of the same enzyme in solution at pH 7. The activity of the enzyme in aqueous solution is the function of pH. The increased residual activity might be due to the unfolding of the adsorbed protein above the isoelectric point (which is 5) as a result of electrostatic repulsion, as previously reported by Norde, (1986). Interestingly, hyperactivation of the enzyme was found within the studied pH range, which might be due to the same reason as mentioned in Section 4.1.4.1. Moreover, the protein loading remains constant between the pH range of 6.5 and 7, then it increases at pH 7.5 and again remains constant till pH 8. It is clear from Figure 4.12 that the protein adsorption was influenced by the change in pH value, indicating that the dominating adsorption forces were electrostatic interactions (not hydrophobic interactions).
Figure 4.12: Effect of buffer pH on immobilization efficiency of plain cell extract of ADH T: Black bars represent residual activity and white bars denote protein loading. Immobilization conditions: 170 units of plain cell extract of ADH T, 500 mg of non-porous plain glass beads, 1 ml of 50 mM PO₄-buffer (143 mM ionic strength), 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

In the next step, the effect of PO₄-buffer concentration on immobilization efficiency of plain cell extract of ADH T was studied by varying the buffer concentration in a range of 20 mM to 200 mM. As depicted in Figure 4.13, the residual activity increases with the increase in buffer concentration from 20 mM to 100 mM and then it decreases. The increase in residual activity till 100 mM buffer concentration might be attributed to structural changes of the enzyme with increase in ionic strength, which was retained even after dissolving the enzyme in aqueous solution for activity measurement. At concentrations much higher than the practical range of 20 mM to 100 mM, the decrease in residual activity might be owing to the conformational changes in enzyme caused by the substantial increase in ionic strength leading to loss of enzyme activity. Similar effects of decrease in diastase activity with increasing salt concentration were observed by Bajpai and Sachdeva, (2002). But the residual activity was still higher than the initial activity of the enzyme. The protein loading increases with the increase in buffer concentration till 50 mM and then it decreases, which might be due to the occupation of the surface of the glass beads with salt, thereby hindering the protein adsorption.
Figure 4.13: Effect of buffer concentration on immobilization efficiency of plain cell extract of ADH T: Black bars represent residual activity and white bars denote protein loading. Immobilization conditions are the same as mentioned against Figure 4.13, except 1 ml of PO\textsubscript{4}-buffer (pH 7).

Figure 4.14: Effect of buffer type on immobilization efficiency of lyophilized LBADH (A) and lyophilized ADH T (B): Black bars represent residual activity and white bars denote protein loading. Immobilization conditions: 2 mg of lyophilized LBADH or lyophilized ADH T (corresponding to 170 units), 500 mg of non-porous plain glass beads, 1 ml of buffer (pH 7), 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.
Further, the effect of type of buffer on immobilization efficiency of the lyophilized LBADH and the lyophilized ADH T was evaluated. The enzymes were immobilized with different buffers at pH 7. As seen in Figures 4.14A and B, the residual activity and the protein loading of the immobilized LBADH and the immobilized ADH T followed similar trend. However, it was noticeable to see the extent to which the buffer affected the residual activity depends on the source of the enzyme and the enzyme preparation. The residual activity of the immobilized LBADH and the immobilized ADH T remained almost constant for three buffers namely Tea, PO$_4$, and Mops but not for Tris. Hyperactivation of LBADH and ADH T was found for all four buffers except for Tris-buffer in case of ADH T (Figures 4.14A and B). Relatively low residual activity of LBADH and ADH T for Tris-buffer might be due to the interaction of some groups on the protein with the combination of the added Tris-buffer and PO$_4$-buffer originally present in the lyophilized enzyme powder. In general, the lyophilized LBADH retained higher residual activity as compared to the lyophilized ADH T after immobilization. Still the protein loading of LBADH and ADH T was same. Because these two lyophilized enzymes contained 50 mM of PO$_4$-buffer (pH 7), which was used for lyophilization of these enzymes, the results of the immobilization efficiency might be due to the combination effects of the buffers.

In this context to investigate the effect of single buffer on the immobilization efficiency, the plain and heat-treated cell extracts of ADH T were immobilized with the same buffers as before (Figures 4.15A and B). On the contrary to lyophilized LBADH and lyophilized ADH T, hyperactivation of the plain and heat-treated cell extract of ADH T was found with all the buffers after immobilization. The protein loading of plain and heat-treated cell extracts was almost constant with all buffers except Mops. The reason for slightly lower protein loading with Mops is not known.

Indeed, comparison of the residual activities of immobilized lyophilized ADH T (lyophilized ADH T was obtained by lyophilizing the heat-treated cell extract of ADH T with sucrose 5 times the protein amount on weight basis) with immobilized heat-treated cell extract of ADH T suggested that the lyophilization process might have led to a loss of enzyme. Although the lyophilized ADH T contained sucrose 5 times the protein amount (w/w) as compared to no sucrose in heat-treated cell extract, the residual
activity of the latter was still higher as compared to the former (Figure 4.13B and Figure 4.15B).

Figure 4.15: Effect of buffer type on immobilization efficiency of plain cell extract of ADH T (A) and heat-treated cell extract of ADH T (B): Black bars represent residual activity and white bars denote protein loading. Immobilization conditions are the same as mentioned against Figure 4.14.

### 4.1.5 Effect of amount of added protein on immobilization efficiency

The immobilization efficiency of lyophilized LBADH and lyophilized YADH was studied by varying the protein amount from 0.001 mg/mg to 0.016 mg/mg non-porous plain glass beads. The residual activity of LBADH remained almost constant with the increase in protein loading, while minor decrease in residual activity of the YADH was found only at 0.001 mg/mg non-porous plain glass beads (Figures 4.16A and B). As
YADH is a sensitive alcohol dehydrogenase (Ooshima, et al. 1981), the adsorption of the enzyme on the support might lead to increase in structural changes, with decrease in surface coverage during the adsorption of low amounts of protein (Soderquist and Walton, 1980). Owing to these structural changes, the residual activity of YADH decreased at 0.001 mg/mg of non-porous glass beads. The protein loading on the non-porous plain glass beads increased with the increase in added protein due to the adsorptive deposition of the protein on non-porous plain glass beads. Interestingly, the percentage of protein loaded on the non-porous plain glass beads relative to the initially added protein remained almost constant for LBADH (57 %) and YADH (38 %) as seen from the linear rise in protein loading in Figures 4.13A and B. This indicates that YADH under the given immobilization conditions had a lower affinity of the non-porous plain glass beads than LBADH. A saturation of the surface of the non-porous glass beads with the protein was not found in the studied range.
Figure 4.16: Effect of amount of added protein on immobilization efficiency of lyophilized LBADH (A) and lyophilized YADH (B): (□) represents residual activity and (□) denotes protein loading. Immobilization conditions: 500 mg of non-porous plain glass beads, 1 ml of Tea-buffer (pH 7) with 1 mM of MgCl₂ for LBADH, 1 ml of 100 mM PO₄-buffer (pH 7.84) for YADH, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

4.1.6 Effect of supports on immobilization efficiency

The residual activity and protein loading of various supports was studied with and without addition of sucrose. The results are shown in Table 4.1. Evidently, sucrose addition enhanced the residual activity and decreased the protein loading of YADH on all non-porous glass beads for the same reason as mentioned in Section 4.1.4.1. Assuming all support particles to be spherical, the ratio of the surface area of the support to the total available area for adsorption (support and beaker used during the drying process) was calculated. For example, in case of non-porous plain glass beads this ratio was found to be 0.7. It is implied that, if the affinity of the enzyme towards the support particles and the inner wall of the beaker were equivalent, then 70 % of protein solution (which amounted to 0.0056 mg protein/mg of support) would be adsorbed on the support and the rest of about 30 % would be adsorbed on the inner wall of the beaker. The calculated amount of protein loading in mg/mg of support was termed as theoretical protein loading. For all the non-porous glass beads the experimental protein loading was lower than the theoretical values. In all cases, in the beaker after drying dry enzyme powder together with salt crystals was found that did not adsorb either on the support or on the inner wall of the beaker. This clearly indicates that the surface properties of the support material also played an important role.

In order to evaluate the effect of the surface properties, glass beads were treated with hydrophobic and hydrophilic reagents as described in Section 3.2.2. Comparison between the hydrophobic and hydrophilic glass beads revealed that the hydrophobic glass beads had low protein loading but higher residual activity in contrast to hydrophilic glass beads. In this connection, the effect of a varying degree of hydrophilicity of glass beads on the immobilization efficiency was studied. It was found
that the residual activity was almost same for all the hydrophilic glass beads but the protein loading increased with the increase in hydrophilicity of the glass beads in the following order: hydrophilic glass beads > acid washed glass beads (Sigma-Aldrich Chemie GmbH) > acid unwashed glass beads (Sigma-Aldrich Chemie GmbH) ≥ plain glass beads (B. Braun Biotech International GmbH) in the particle size range of 0.151 to 0.30. This observation is in accordance with Norde, (1986) and Barroug, et al. (1989), who reported that the electrostatic forces are known to control the adsorption of protein on hydrophilic surfaces and the protein loading increases with the increase in hydrophilicity of the support. As previously reported by Bailey and Cho, (1983), the residual activity and the protein loading increases with decrease in particle size of the support. Similar observation was found comparing the acid washed and unwashed glass beads based on their particle size.

Apart from the non-porous glass beads, immobilization of YADH was also conducted on other organic and inorganic supports: such as Celite 545, Celite 503, Polyamide, Accurel, Silica gel 25 Å, and Silica gel 60 Å at the optimal immobilization conditions with and without addition of sucrose. The results are shown in Table 4.1. Addition of sucrose enhanced the residual activity and decreased the protein loading of all the supports for the same reason as mentioned in Section 4.1.4.1. Among these various supports, Celite 545 retained the highest protein loading of 68.8 % relative to the theoretical protein loading without sucrose addition. Between the two Celites with the same surface characteristic the protein loading increased with decrease in particle size. Comparison between the two organic supports i.e., Polyamide and Accurel revealed a minor difference in protein loading, whereas the residual activity with and without sucrose addition was higher for Accurel, which is more hydrophobic than polyamide. Among the two Silica gel supports one might expect higher protein loading on Silica gel 60 Å having larger pore diameter than Silica gel 25 Å. However, the protein loading was same.

The effect of various surface treated glass beads on immobilization efficiency of the plain cell extract of LBADH was investigated with and without sucrose addition as depicted in Table 4.2. Addition of sucrose increased the residual activity and decreased the protein loading for similar reason as mentioned in Section 4.1.4.1. Interestingly, similar to YADH, the protein loading of LBADH was higher on hydrophilic glass beads
than on plain and hydrophobic glass beads (Table 4.2). The residual activity and protein loading of LBADH followed a similar trend for various surface treated glass beads as with YADH.

In summary, it can be said that the residual activity and the protein loading of ADH enzyme was dependent not only on the nature and the particle size of the support but also on the source of the enzyme.

Table 4.1: Effect of supports on immobilization efficiency of lyophilized YADH

<table>
<thead>
<tr>
<th>Support</th>
<th>Treatment</th>
<th>Particle size [mm]</th>
<th>Residual activity [%]</th>
<th>Theoretical protein [a] loading [mg protein/mg support]</th>
<th>Experimental protein loading [mg protein/mg support]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain glass beads (B. Braun Biotech GmbH)</td>
<td>Without sucrose</td>
<td>0.25 – 0.30</td>
<td>69</td>
<td>0.0056</td>
<td>0.0030</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>80</td>
<td></td>
<td>0.0027</td>
</tr>
<tr>
<td>Hydrophobic glass beads</td>
<td>Without sucrose</td>
<td>0.25 – 0.30</td>
<td>81</td>
<td>0.0056</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>89</td>
<td></td>
<td>0.0021</td>
</tr>
<tr>
<td>Hydrophilic glass beads</td>
<td>Without sucrose</td>
<td>0.25 – 0.30</td>
<td>70</td>
<td>0.0056</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>80</td>
<td></td>
<td>0.0032</td>
</tr>
<tr>
<td>Acid washed glass beads</td>
<td>Without sucrose</td>
<td>0.151 – 0.212</td>
<td>66</td>
<td>0.0057</td>
<td>0.0033</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>78</td>
<td></td>
<td>0.0031</td>
</tr>
<tr>
<td>Acid washed glass beads</td>
<td>Without sucrose</td>
<td>0.425 – 0.600</td>
<td>57</td>
<td>0.0054</td>
<td>0.0030</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>70</td>
<td></td>
<td>0.0027</td>
</tr>
<tr>
<td>Acid unwashed glass beads (Sigma-Aldrich chemie GmbH)</td>
<td>Without sucrose</td>
<td>0.151 – 0.212</td>
<td>72</td>
<td>0.0057</td>
<td>0.0031</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>82</td>
<td></td>
<td>0.0028</td>
</tr>
<tr>
<td>Acid unwashed glass beads (Sigma-Aldrich)</td>
<td>Without sucrose</td>
<td>0.425 – 0.600</td>
<td>62</td>
<td>0.0054</td>
<td>0.0028</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>72</td>
<td></td>
<td>0.0025</td>
</tr>
</tbody>
</table>
Table 4.2: Effect of surface-treated glass beads on immobilization efficiency of plain cell extract of LBADH

<table>
<thead>
<tr>
<th>Support</th>
<th>Treatment</th>
<th>Particle size [mm]</th>
<th>Residual activity [%]</th>
<th>Theoretical protein [a] loading [mg protein/mg support]</th>
<th>Experimental protein loading [mg protein/mg support]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celite 545</td>
<td>Without sucrose</td>
<td>0.01 – 0.02</td>
<td>59</td>
<td>0.0080</td>
<td>0.0055 0.0043</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Support</td>
<td>Treatment</td>
<td>Particle size [mm]</td>
<td>Residual activity [%]</td>
<td>Theoretical protein [a] loading [mg protein/mg support]</td>
<td>Experimental protein loading [mg protein/mg support]</td>
</tr>
<tr>
<td>Celite 503</td>
<td>Without sucrose</td>
<td>&lt;0.1</td>
<td>53</td>
<td>0.0079</td>
<td>0.0047 0.0042</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyamide</td>
<td>Without sucrose</td>
<td>0.05 – 0.16</td>
<td>58</td>
<td>0.0078</td>
<td>0.0025 0.0022</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accurel</td>
<td>Without sucrose</td>
<td>&lt;1.5</td>
<td>68</td>
<td>0.0074</td>
<td>0.0023 0.0020</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silica gel 25 Å°</td>
<td>Without sucrose</td>
<td>0.07 – 0.14</td>
<td>48</td>
<td>0.0076</td>
<td>0.0027 0.0022</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silica gel 60 Å°</td>
<td>Without sucrose</td>
<td>0.40 – 0.63</td>
<td>21</td>
<td>0.0065</td>
<td>0.0021 0.0018</td>
</tr>
<tr>
<td></td>
<td>With sucrose</td>
<td></td>
<td>37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Theoretical protein loading calculated based on the theoretical protein content in the enzyme solution.
4.2 Evaluation of batch gas-phase reactors

4.2.1 Selection of a batch reactor

Selection of a batch reactor among the various modified types, as shown in Section 3.3.3 was done based on their performances evaluated on the basis of some parameters.

![Figure 4.17: Effect of stirring speed on absolute pressure in different batch reactors: (■) represents Frings turbine, II\textsuperscript{nd} (□) denotes Frings turbine I\textsuperscript{st}, and (◊) represent hollow shaft reactor. 50 ml of saturated ammonium sulfate salt solution was used.](image)

The effect of stirring speed on absolute pressure is shown in Figure 4.17. Clearly, there was no change in the absolute pressure until a specific stirring speed was reached and then it started decreasing almost linearly. This was due to the increase in the intensity of the vacuum created in the column of the reactor due to increase in stirring speed. In fact, the intensity of the decrease in absolute pressure with the stirring speed was maximum with Frings turbine II\textsuperscript{nd} as compared to Frings turbine I\textsuperscript{st} as well as the hollow shaft reactor. In Frings turbine, vacuum was created in the small gap between the edges of the rotor and the stator due to the rotation of the rotor in the stator. This was more intensive.

---

<table>
<thead>
<tr>
<th>Hydrophilic glass beads</th>
<th>Without sucrose</th>
<th>With sucrose</th>
<th>0.25 – 0.30</th>
<th>86</th>
<th>129</th>
<th>0.0028</th>
<th>0.0021</th>
<th>0.0019</th>
</tr>
</thead>
</table>

[a] Theoretical protein loading was calculated using the average size of the support.
with Frings turbine II\textsuperscript{nd} than in Frings turbine I\textsuperscript{st}. This might be due to the opposite direction of the rotor wings (anticlockwise) and stator tooth (clockwise) in case of the former; whereas the rotor and the stator were in the same direction (clockwise) in case of the latter. The decrease in absolute pressure in the column of the hollow shaft was quite small as compared to Frings turbine I\textsuperscript{st} and II\textsuperscript{nd} as the stirring disc was not able to produce sufficient driving force for the convective flow of the air, and the intensity of the eddies created during the stirring at the cut edges in front of the holes of the stirring disc (connected to the hollow shaft) was too low.

![Graph](image)

Figure 4.18: Effect of stirring speed on air flow rate in different batch reactors: (■) represents Frings turbine II\textsuperscript{nd}, (□) denotes Frings turbine I\textsuperscript{st}, and (◊) represents hollow shaft batch reactor. 50 ml of saturated ammonium sulfate salt solution was used.

In the next step, the effect of stirring speed on air flow rate in various modified batch reactors were evaluated using a gas flow meter. As shown in Figure 4.18, the “measurable” flow of the gas began only after reaching the critical stirring speeds. Actually the air flow began slightly before the critical stirring speed, but the flow could not be detected due to the instrumental resistance of the gas flow meter. Clearly, the measurable air flow began at a slightly lower stirring speed in case of Frings turbine II\textsuperscript{nd} than for Frings turbine I\textsuperscript{st}, whereas the measurable air flow rates began at relatively high stirring speed with the hollow shaft reactor. Then the air flow rates increased with the increase in stirring speed owing to increase in convective flow of the air. The driving force for the air suction was the intensity of the vacuum created in the reactor column.
during stirring. Because the intensity of the vacuum created during stirring in the column of Frings turbine II\textsuperscript{nd} was higher than in Frings turbine I\textsuperscript{st} as well as the hollow shaft reactor, the overall air flow rate performance of Frings turbine II\textsuperscript{nd} was the best. It is observable that the air flow rate in both Frings turbines followed a specific pattern with the increase in stirring speed. Initially it followed a steep and linear increase, and then it rose slowly till a specific stirring speed was reached, after that it again continued to increase steeply and linearly. The exact reason for this trend is unknown. The change in air flow rate with increasing stirring speed was very low for hollow shaft reactor.

![Figure 4.19: Effect of stirring speed on air flow rate in Frings turbine II\textsuperscript{nd} at different conditions: (□) represents 40 ml filling volume, (■) denotes 50 ml filling volume, and (♦) represents 40 ml filling volume and the reactor equipped with 2 POM rings and membrane filter paper.](image)

Thus, the performance of Frings II\textsuperscript{nd} was the best, as evaluated on the basis of the above parameters. Further studies were conducted with this reactor to examine the air flow rate under actual experimental conditions. As a first step to enhance the air flow rate, the volume of the salt solution was reduced from 50 ml to 40 ml. As depicted in Figure 4.19, the air flow rate began at much lower stirring speed with the decrease in volume of the salt solution. This was attributed to a smaller resistance to the movement of the rotating rotor in the salt solution, which in turn enhanced the overall air flow rate at 40 ml filling volume compared to 50 ml filling volume at all stirring speeds. The specific air flow rate pattern with increasing stirring speed was even more clearly visible at 40
ml filling volume than at 50 ml filling volume. When the stator of the reactor was equipped with two POM (polyoxymethylene material) rings and the membrane filter paper (to simulate the experimental conditions), the specific air flow pattern with increasing stirring speed was no longer noticed. As POM rings and the membrane filter paper created resistance to the flow of the air, the air flow began at slightly higher stirring speed and the increase in air flow rate with the increase in stirring speed was lower than at 40 ml filling volume without POM rings and membrane filter paper.

4.2.2 Functionality of the selected batch reactor

In the initial step, same reactions were carried out with two Frings turbine II to check the reproducibility of the results. The conversion of hexanal to hexanol was carried out using immobilized YADH preparation at 30 °C. As depicted in Figure 4.20, in both reactors the increase in hexanal conversion in liquid and gas-phase with respect to time was the same. Thus, the reproducibility of the same reaction between the two batch reactors was verified. The maximum conversion at the end of reaction was about 45 % in the liquid-phase and 30 % in the gas-phase.

![Figure 4.20: Conversion-time profile of hexanal to hexanol in enzymatic gas-phase reaction using batch reactor: (□) represents conversion in liquid-phase and (■) denotes conversion in gas-phase. Reaction conditions: 40 ml of saturated ammonium sulfate salt solution (RH 80.63 %), 100 mg of immobilized YADH was taken, 100 µl of hexanal (800 µmol), 1 ml of ethanol (17 mM), 30 °C temperature. Immobilization conditions: 8](image-url)
mg of lyophilized YADH, 500 mg of non-porous plain glass beads, 1 ml of 100 mM PO₄-buffer (pH 7.84), cofactor-to-protein molar ratio 1.95, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

In the next step, the reduction of acetophenone to (R)-phenylethanol was carried out with 100 mg and 200 mg immobilized LBADH preparations. The experiments were run in duplicates at 30 °C using saturated ammonium sulfate as the substrate reservoir. Interestingly, the conversion of the substrate was almost doubled with the increase in enzyme loading from 100 mg to 200 mg (Figures 4.21A and B).

![Figure 4.21: Conversion-time profile of acetophenone to (R)-phenylethanol in enzymatic gas-phase reaction using batch reactor: (□) represents conversion with 100 mg and (■) denotes conversion with 200 mg of immobilized LBADH preparation. Reaction conditions: 40 ml of saturated ammonium sulfate salt solution (RH 80.63 %), 100 µl of acetophenone (850 µmol), 250 µl of isopropanol (3.25 mmol), temperature 30 °C. Immobilization condition: 2 mg of lyophilized LBADH, 500 mg of non-porous plain glass beads, 1 ml of distilled water, cofactor-to-protein molar ratio 1.25, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

It should be noted here that a saturated ammonium sulfate solution having a 80.63 % RH was used as substrate reservoir because of the corrosion effect of chloride ions present in some salt solution having low RH, which are generally used as substrate reservoir. In future, other non-corroding saturated salt solutions may be used.
In the above study, due to the high RH (80.63 %) water mediated inactivation of the enzyme might have led to low conversion with both enzymes. Moreover, the exact concentration of the compounds in the gas and liquid-phase could not be determined due to the difficulties in analysis as mentioned in Section 3.2.11.1. Therefore, in order to determine the influence of immobilization and gas-phase reaction parameters on the immobilized enzyme preparation, a continuous gas-phase reactor was used.

4.3 **Optimization of continuous enzymatic gas-phase reaction parameters**

4.3.1 **Effect of total gas flow rate on initial substrate conversion and initial reaction rate of immobilized enzyme preparation**

Figure 4.22: Initial substrate conversion and initial reaction rate versus total gas flow rate or molar flow of acetophenone (A) and residence time (B) of immobilized lyophilized LBADH: ( ) represents initial conversion and (□) denotes initial reaction rate. Gas-phase reaction conditions: Acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 60, isopropanol activity 0.116, temperature 60 °C, water activity 0.65, 500 mg of immobilized enzyme preparation. Immobilization conditions: 2 mg (corresponding to 170 units) of lyophilized LBADH, 500 mg of non-porous plain glass beads, 1 ml of distilled water, cofactor-to-protein molar ratio 1.25, 4 °C
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temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

The effect of total gas flow rate on the initial substrate conversion and the initial reaction rate was studied by varying the inlet gas flow rate from 15 ml/min to 88.5 ml/min. As a consequence the molar flow of acetophenone varied from 0.43 µmol/min to 2.53 µmol/min. As depicted in Figure 4.22A, the initial substrate conversion was constant in the range of the molar acetophenone flow rate from 0.43 µmol/min to 1.96 µmol/min and then it started decreasing. In contrast, the initial reaction rate increased with the increase in molar acetophenone flow up to 2.24 µmol/min and then it remained almost constant. The constant values of initial conversions between 0.43 µmol/min and 1.96 µmol/min molar flow of acetophenone indicates that the reaction was thermodynamically limited and therefore the initial conversion was almost constant in this range. As the total gas flow rate was further increased, the residence time (It is defined as the ratio of the reactor volume to the feed rate.) of the substrates in the bioreactor was too short for the completion of the reaction and therefore the initial conversion decreased (Figure 4.22B). Erable, et al. (2004) and Lamare, et al. (1997) observed similar effects of molar flow of substrate for dehalogenation reaction using Rhodococcus erythropolis and transesterification using Fusarium solani pisi cutinase, respectively.

4.3.2 Effect of water on initial reaction rate and half-life of immobilized enzyme preparation

In this study, the effect of water on the initial reaction rate and the half-life (Half-life is defined as the time in which the conversion reduces to one-half of its maximum value.) of the immobilized enzyme preparation was studied in the gas-phase reaction condition at 60 °C by varying the water activity (Water activity is defined as the ratio of partial pressure of water in the gas entering the bioreactor to the saturation pressure of the pure water at the same temperature.). Interestingly, for immobilized LBADH and immobilized ADH T preparations, a critical water activity of 0.35 was needed for the enzyme to become active, as evident in Figures 4.23A and B. Almost all the previous researchers working with biocatalyst in non-conventional media such as organic solvent or gas-phase found similar effects of the critical water activity on the biocatalyst
performance (Erable, et al. 2004; Grizon, et al. 2004; Goubet, et al. 2002; Maugard, et al. 2001; Aldercreutz, 1993; Deetz and Rozzel, 1988 and Skujins and McLaren, 1967). The critical water activity varies with the source of the enzyme and its preparation resulting from addition of buffers, sucrose, etc., (Ross and Schneider, 1991 and Jones, 1986). Then the initial reaction rate increased almost linearly with the increase in water activity up to 0.55. This might be related to an increase in flexibility of the enzyme as water acts as a plasticizer, favoring the intramolecular movement of the protein, enhancing the catalytic activity, and it facilitates weak interaction for the binding of substrate at the active site of the enzyme (Hartsough and Merz, 1993; Zaks and Klibanov, 1988 and Klibanov, 1989). At the same time, the thermo-stability of the immobilized enzyme preparations decreased almost linearly with the increase in water activity owing to the thermo-inactivation of enzyme mediated by water (Turner, et al. 1995; Rupley and Careri, 1991; Klibanov and Ahern, 1987; Klibanov, 1986 and Kuntz and Kauzmann, 1974). It is interesting to note that the initial reaction rate was lowest and the thermo-stability of the enzyme was highest at the critical water activity. Beyond the critical water activity, the thermo-stability of the enzyme started decreasing. This might be due to the increase in amount of water on the immobilized enzyme preparations. Similar observations were made with other enzymes by previous researchers such as Erable, et al. (2004); Goubet, et al. (2002); Yang and Russell, (1996b) and Barzana, et al. (1989). As shown in Figure 4.20, although the thermostability of both the enzymes was high below the water activity of 0.55, but the initial reaction rate with both the enzymes was higher above the water activity of 0.55. From the economic point of view, an optimum water activity for LBADH as well as for ADH T was 0.55.

![Graph A](image1.png)

![Graph B](image2.png)
Figure 4.23: Effect of water activity on initial reaction rate and half-life of immobilized lyophilized LBADH (A) and immobilized plain cell extract of ADH T (B): (□) denotes initial reaction rate and (▏▏) represents half-life. Gas-phase reaction conditions: Flow rate 35 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 60, isopropanol activity 0.116, 60 °C temperature, 150 mg of immobilized enzyme preparation. Immobilization conditions: 170 units of lyophilized LBADH or plain cell extract of ADH T, 500 mg of non-porous plain glass beads, 1 ml of distilled water for LBADH and 1 ml of 50 mM PO₄⁻-buffer (pH 7) for ADH T, cofactor-to-protein molar ratio 1.25 for LBADH and 1.78 for ADH T, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

4.3.3 Effect of additives on initial reaction rate and half-life of immobilized enzyme preparation

4.3.3.1 Buffers

Figure 4.24: Effect of buffer pH on initial reaction rate and half-life of immobilized plain cell extract of ADH T: (□) denotes initial reaction rate and black bars represent half-life. Gas-phase reaction conditions: Flow rate 25 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 45, isopropanol activity 0.068, temperature 40 °C, water activity 0.5, 150 mg of immobilized enzyme preparation. Immobilization conditions: 170 units of plain cell extract of ADH T, 500 mg of non-
porous plain glass beads, 1 ml of 50 mM PO₄-buffer (143 mM ionic strength), cofactor-to-protein molar ratio 1.78, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

The effect of pH value of the buffer used for immobilization on the initial reaction rate and the half-life was studied in the gas-phase reaction. The plain cell extract of ADH T was immobilized with the 50 mM PO₄-buffer (143 mM ionic strength) within a pH range from 6.5 to 8. As depicted in Figure 4.24, the initial reaction rate was almost constant at a pH range of 6.5 to 7 and then it started decreasing. The thermo-stability of the immobilized enzyme preparation followed a bell shaped curve with a maximum at 7. At different pH values the net charge on the protein is different and the interaction of charged amino acids with the support will also be different. In addition, according to the pH memory effect reported by Zaks and Klibanov, (1988 and 1985), the pH of the last aqueous solution from which the enzyme is recovered affects the pH value of the microenvironment of the biocatalyst in a non-aqueous media. The optimum pH of the enzyme was 7. The net result of the aforesaid phenomenon might be influencing the initial reaction rate and thermo-stability of the ADH T.

Figure 4.25: Effect of buffer concentration on initial reaction rate and half-life of immobilized plain cell extract of ADH T: (□) denotes the initial reaction rate and black bars represent the half-life. Gas-phase reaction conditions are the same as mentioned against Figure 4.16. Immobilization conditions are the same as mentioned against Figure 4.21, except 1 ml of PO₄-buffer (pH 7).
The effect of buffer concentration on the initial reaction rate and half-life of the immobilized enzyme preparation was examined in the second step. The plain cell extract of ADH T was immobilized with different concentrations of PO$_4$-buffer in the range of 20 mM to 200 mM. It was found that the initial reaction rate decreased with increase in buffer concentration, while the thermo-stability of the preparation increased with the increase in buffer concentration up to 50 mM and then it started decreasing (Figure 4.25). With the increase in buffer concentration the ionic strength of the buffer was an increased and therefore the protein hydration was increased, resulting in increase in thermo-inactivation of the protein mediated by water. Indeed, the above mentioned effects resulted in a drastic decrease in thermo-stability of the immobilized enzyme preparation at buffer concentrations higher than 50 mM, which in turn might be affecting the initial reaction rate.

In the third step, the effect of the type of the buffer used for immobilization on the initial reaction rate and the half-life of the immobilized lyophilized LBADH and immobilized lyophilized ADH T preparations was studied. The initial reaction rate and the half-life of both immobilized enzyme preparations followed a similar trend. The gas-phase reaction with immobilized LBADH was carried out at 60 °C and with immobilized ADH T at 40 °C. Therefore, only the qualitative evaluation of the effect of buffer is described here. As shown in Figures 4.26A and B, the initial reaction rate of both immobilized enzyme preparations was almost similar for all the buffers, except for Tris-buffer. The half-life of immobilized enzyme preparations were low for all the organic buffers such as Tris, Mops, and Tea and high for inorganic one i.e. PO$_4$-buffer. Sears, et al. (1994), observed that the subtilisin enzyme lyophilized from organic buffer deactivated faster in dimethyl formamide than that from the inorganic buffer. This means there was some process taking place during the enzyme immobilization, which was influenced by the buffer.

In order to investigate the reason of it, various buffer solutions with and without addition of enzyme preparation (which includes enzyme in a lyophilized or cell extract, cofactor, and support) were subjected to the immobilization conditions (as described in Section 3.2.3). All buffer solutions were prepared at 25 °C. At this temperature the buffer solution had an initial pH value (which was set for the immobilization). Addition
of enzyme preparation resulted in a minor change (0.05 to 0.10) in pH value in each of
the buffer solution. Then as described in the immobilization procedure, the buffer
solutions with and without enzyme preparation were taken to a thermo-constant
chamber of 4 °C for mixing the enzyme preparation. The pH value of all the buffer
solutions with and without enzyme preparation was checked after 30 min. Surprisingly,
in case of all the buffer solutions with and without enzyme preparation, a dramatic rise
in pH value was noted, except for PO$_4$-buffer where the rise in pH value was minor
(0.05 to 0.1). This is shown in the Figures 4.27A and B. This is in agreement with the
temperature dependent pH shift observed for these buffers. All the buffer solutions
retained their initial pH value after some time at 25 °C (data not shown). As soon as the
temperature was reduced from 25 °C to 4 °C, there was again a rise in pH value.
Increasing the duration of exposure from 30 min to 2 h at 4 °C resulted in minor rise in
the pH value of all the buffer solutions. This change in initial buffer pH value with and
without enzyme preparation was in the order as follows: Tris (pH 7 and 8) > Tea (pH 7)
> Mes (pH 6) > Mops (pH 7) > PO$_4$ (pH 7), as evident from Figures 4.27A and B. In
this context, Lam, et al. (1996) reported that by preventing the change in buffer pH
during lyophilization by replacing succinate with glycolate buffer, whose pH does not
change significantly with temperature, the stability of the lyophilized interferon can be
improved.

According the Hofmeister series the stabilizing effect of the anions on the protein is in
the following order: Chloride > phosphate > sulfonate (Hofmeister, 1888). These anions
stabilize the protein by strongly interacting with water molecules than water with itself
and therefore were capable of breaking water-water hydrogen bonds.

In addition, even though the water activity was 0.5, the amount of water associated with
each enzyme preparation depends on the hygroscopic nature of the buffer salts.
According to Ross and Schneider, (1991), the hygroscopicity of the buffer defines the
water present in the thin film around the enzyme. Some of the buffer salts such as Tea
and Tris used in this study are known to be highly hygroscopic as compared to PO$_4$.
From previous studies of Ferloni, (2004), it is clear that the amount of water associated
with the immobilized enzyme prepared with these hygroscopic buffers such as Tris and
Tea would be high as compared to PO$_4$. It is evident from the previous findings of
Klibanov and Ahern, (1987) that the thermo-stability of the enzyme preparation depends
on the amount of water present. Therefore, the thermo-stability of the immobilized enzyme preparations prepared with these hygroscopic buffers (Tris and Tea) was low.

In case of lyophilized and cell extracts, enzyme residual activity measured in aqueous solution was not directly proportional to the corresponding decrease in conversion observed in the gas-phase reaction for the immobilized enzyme prepared using Tris-buffer. This might be due to the conformational changes in the enzyme and/or the cofactor and/or both resulting in the low thermo-stability of immobilized enzyme preparation in the gas-phase reaction medium. This conformational changes might be reversible, when the immobilized enzyme preparation were dissolved in the aqueous solution for activity measurement (data not shown).
Figure 4.26: Effect of type of buffers used for immobilization on initial reaction rate and half-life of the immobilized lyophilized LBADH (A) and immobilized lyophilized ADH T (B): (□) denotes initial reaction rate and black bars represent half-life. Gas-phase reaction conditions for LBADH: Flow rate 88.5 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 60, isopropanol activity 60, isopropanol activity 0.116, temperature 60 °C, water activity 0.55, 120 mg of immobilized enzyme preparation. Gas-phase reaction conditions for ADH T: Flow rate 15 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 45, isopropanol activity 0.068, temperature 40 °C, water activity 0.5, 250 mg of immobilized enzyme preparation. Immobilization conditions: 170 units of lyophilized LBADH or lyophilized ADH T, 500 mg of non-porous glass beads, 1 ml of 50 mM specific buffer, cofactor-to-protein molar ratio 1.25 for LBADH and 1.78 for ADH T, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.
Figure 4.27: Effect of change in buffer pH with temperature for plain buffer solutions (A) and buffer solution containing enzyme in lyophilized or cell extract preparation (B): white bars denote initial pH at 25 °C, dark grey bars represent pH after 30 min of exposure to 4 °C, light grey bar denote pH after 2 h of exposure to 4 °C and black bars represent pH at 90 % volume, while bars with waves in between represent 50 % volume and shaded bars denote 15 % volume of the solution (non-temperature compensated probe).

Figure 4.28: Effect of type of buffers used for immobilization on initial reaction rate and half-life of immobilized plain cell extract of ADH T (A) and immobilized heat-treated cell extract ADH T (B): (□) denotes initial reaction rate and black bars represent half-
life. Gas-phase reaction conditions: Flow rate 15 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 45, isopropanol activity 0.068, temperature 40 °C, water activity 0.5, 150 mg of immobilized enzyme preparation. Immobilization conditions: 170 units of plain or heat-treated cell extract of ADH T, 500 mg of non-porous glass beads, 1 ml of 50 mM specific buffer, cofactor-to-protein molar ratio 1.78, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

Since the lyophilized LBADH and lyophilized ADH T preparations originally contained 50 mM PO$_4$-buffer (pH 7) the above results (in Figures 4.28A and B) might be due to the combination effect of PO$_4$-buffer with the added buffer. Hence, in order to understand the effect of a single buffer on the initial reaction rate and the half-life of the enzyme preparations, immobilization of plain and heat-treated cell extracts of ADH T was performed with a specific buffer. The initial reaction rate was almost the same for all buffers, whereas the half-life followed almost similar trend as with lyophilized LBADH and lyophilized ADH T. The reason of it is already described in the previous paragraphs.

As depicted in Figures 4.28A and B, the half-life of the immobilized plain cell extract preparation was better as compared to the immobilized heat-treated cell extract (fairly purified) preparation, which might be due to the stabilizing effect of other proteins present in the immobilized plain cell extract preparations (Ariga, et al. 1996).

A marked difference between the performance of the plain and the heat-treated cell extracts was found only in the gas-phase reaction. However, a slight difference in the residual activity of the immobilized plain and immobilized heat-treated cell extracts was observed when the enzyme activity was determined in aqueous solution (Figures 4.15A and B). In conventional enzymology (aqueous media), rehydration reverses much of the structural damage occurred during dehydration, reported by Triantafyllou, et al. (1997).

### 4.3.3.2 Sucrose and others
The thermo-stability of the immobilized YADH preparations with and without sucrose was determined at 30 °C by measuring the enzyme residual activity at regular intervals. The immobilized enzyme preparations were kept under the RH of 67.9 % and 75.1 %
using saturated salt solutions of potassium iodide and sodium chloride, respectively, as described in Section 3.2.6.2. In general, the immobilized enzyme preparation without sucrose showed a drastic decrease in residual activity with time as compared to with sucrose as shown in Figure 4.29. According to the previous reports of Miroliaei and Nemat-Gorgani, (2001), various processes such as cysteine group oxidation, deamidation, and aggregation can lead to thermo-inactivation of YADH and under all the conditions sucrose is found to be effective in stabilizing YADH via alteration of the protein micro-environment. In addition, at lower RH (67.9 %) the immobilized enzyme preparations with and without sucrose retained better residual activity than at higher RH (75.1 %). This was due to the low water mediated thermo-inactivation of the enzyme preparations (Turner, et al. 1995; Klibanov and Ahern, 1987 and Klibanov, 1986).

Figure 4.29: Residual activity-time profile of immobilized lyophilized YADH with and without sucrose addition: Open symbols (◊) and (□) represents RH 67.9 % and 75.1 % with sucrose addition and filled symbols (♦) and (■) denotes RH 67.9 % and 75.1 % without sucrose addition, respectively. Condition: 30 °C temperature. Immobilization conditions: 4 mg of lyophilized YADH, 500 mg of non-porous plain glass beads, 1 ml of PO₄-buffer (pH 7.84), 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure, with sucrose: sucrose added 5 times the protein amount (w/w).
In this perspective, the effect of addition of various amounts of sucrose on the initial reaction rate and the half-life of the immobilized plain and heat-treated cell extracts of ADH T were studied in the gas-phase reaction. As visualized in Figure 4.30 for both the immobilized enzyme preparations, the initial reaction rate remained almost constant with increase in sucrose. On the other hand, the half-life was increased with the increase in amount of added sucrose till sucrose 5 times the protein amount (w/w) and then it started decreasing. Sucrose is known to bind with the protein in place of water molecules via hydrogen bonding, thereby it decreases the water availability for thermo-inactivation (Allison, et al. 1999). At very high sucrose concentrations, sucrose makes intra-molecular and sucrose-sucrose inter-molecular hydrogen bonds instead of hydrogen bonding with protein. This gives folded and aggregated structure of sucrose molecules that are not enzyme bound, leading to loss of enzyme activity, as reported by Monsan and Combes, (1984). It is presumed that at high sucrose concentrations, the protein unbound sucrose absorbs excess water, which might result in increase in protein mobility, thereby leading to increased initial reaction rate and at the same time decreased half-life due to water mediated thermo-inactivation of the immobilized enzyme preparations. Despite the fact that high sucrose concentrations lead to loss of enzyme activity, decrease in initial reaction rate was not observed due to water mediated increase in flexibility of the protein. Interestingly, in all the above cases the immobilized plain cell extract retained better half-life as compared to the immobilized heat-treated cell extract (fairly purified), which might be due to the thermo-stabilization effects of the other proteins present in the immobilized plain cell extract. The heat-treatment of the plain cell extract with the view of purification of the enzyme might be leading to removal of these stabilizing proteins. The major protein involved in the improvement of the thermo-stability of the plain cell extract is unknown and further investigations are required. Similarly, Ariga, et al. (1996) observed thermo-stabilization of plain cell extract as compared to heat-treated cell extract of β-galactosidase enzyme due to the other proteins present in plain cell extract.

The effect of other stabilizers such as BSA, metal salts (NaCl, KI, MgCl₂, and MnCl₂) and polyols (sucrose and trehalose) on the initial reaction rate and the half-life of the immobilized lyophilized ADH T preparation was studied in the gas-phase reaction at 40 °C. The results are shown in Figure 31. As compared to other stabilizers, the initial reaction rate and the thermo-stability of the immobilized lyophilized preparation were
higher with sucrose. The high initial reaction rate and thermo-stability of the immobilized enzyme preparation was not reached in the gas-phase reaction with the different additives, which might be due to the presence of the Tris-buffer in combination with the PO$_4$-buffer present in the lyophilized ADH T used during the enzyme immobilization.

Figure 4.30: Effect of sucrose addition on initial reaction rate and half-life of immobilized plain cell extract and immobilized heat-treated cell extract of ADH T: (□) denotes initial reaction rate and black bars represent half-life, P stands for plain cell extract of ADH T and HT stands for heat-treated cell extract of ADH T. Gas-phase reaction conditions: Flow rate 15 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 45, isopropanol activity 0.068, temperature 40 °C, water activity 0.5, 150 mg of immobilized enzyme preparation. Immobilization conditions: 170 units of plain or heat-treated cell extract of ADH T, 500 mg of non-porous plain glass beads, 1 ml of 50 m PO$_4$-buffer (pH 7), cofactor-to-protein 3.55, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure, with sucrose: sucrose added 1X, 2X, 5X, and 10X (X stands for times) the protein amount (w/w).
Figure 4.31: Effect of stabilizers on initial reaction rate and half-life of immobilized lyophilized ADH T: (□) denotes initial reaction rate and black bars represent half-life of immobilized ADH T: Gas-phase reaction conditions: Flow rate 15 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 45, isopropanol activity 0.068, temperature 40 °C, water activity 0.5, 250 mg of immobilized enzyme preparation. Immobilization conditions: 2 mg of lyophilized ADH T, 500 mg of non-porous plain glass beads, 1 ml of 50 mM Tris-buffer (pH 7), cofactor-to-protein ratio 3.55, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure, with stabilizers: stabilizers 5X (X stands for times) the protein amount (w/w).

4.3.4 Effect of amount of added protein on initial reaction rate of immobilized enzyme preparation

Barzana, et al. (1989) studied the effect of enzyme content on the initial reaction rate by varying the amount of alcohol oxidase from $5 \times 10^{-6}$ mg/mg to $4 \times 10^{-5}$ mg/mg of controlled pore glass beads and found that the initial reaction rate was a linear function of protein added for the range investigated. Similar attempt was made in this research work, but the range of added protein ($5 \times 10^{-4}$ mg/mg to $1.6 \times 10^{-2}$ mg/mg non-porous plain glass beads) was significantly different than that of Barzana’s study. In the present study, the reaction was carried out at 60 °C with 150 mg of immobilized enzyme preparation. Figure 4.32A shows the results. The initial reaction rate increased almost
linearly with the increase in added protein from 0.0005 mg/mg to 0.002 mg/mg non-porous plain glass beads. This linearity states that the internal diffusion rate of the substrates was higher than the reaction rate. With the further increase in added protein, the initial reaction rate behaved non-linearly with the increase in added protein. In order to understand these behaviors, scanning electron microscopic pictures of the enzyme loaded glass beads were taken. These are shown in Figure 4.32B. The enzyme loaded beads started aggregating at 0.004 mg/mg non-porous plain glass beads. Owing to this, the overall effective enzyme surface area available for reaction was decreased. Hence, the internal diffusion rate of the substrate into the aggregate became limited.

Comparison between Figure 4.16A and Figure 4.32A, at high protein loading on the support, the residual activity of the enzyme measured in aqueous solution remains almost constant due to the solubilization of the protein in the aqueous solution during the enzyme activity measurement, while in gas-phase the diffusion of the gas into the aggregates became limited.

Figure 4.32: Effect of amount of added protein on initial reaction rate of immobilized lyophilized LBADH in gas-phase reaction (A) and scanning electron microscopic pictures of enzyme loaded glass beads (B): (□) denotes initial reaction rate. Gas-phase reaction conditions: Flow rate 35 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 60, isopropanol activity 0.116, temperature 60 °C, water activity 0.55, 150 mg of immobilized enzyme preparation. Immobilization conditions: Specified amount of lyophilized LBADH in mg per mg of glass beads was added, 500 mg of glass beads, 1 ml of distilled water, cofactor-to-protein molar ratio 1.25, 4 °C
temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

4.3.4 Effect of cofactor-to-protein molar ratio on initial reaction rate and half-life of immobilized enzyme preparation

The thermo-stability of the immobilized LBADH preparations with and without cofactor was studied at 60 °C by measuring the residual activity of the immobilized enzyme preparations at regular time intervals. The immobilized enzyme preparations with and without cofactor were weighed beforehand. They were then kept under RH of 63.11 % and 74.5 % using saturated salt solution of potassium iodide and sodium chloride, respectively. The detailed method is described in Section 3.2.6.2. It was observed that the residual activity of the immobilized enzyme preparations decreased with time due to the thermo-inactivation of the enzyme (Figure 4.33). In general, the
immobilized enzyme preparations with cofactor was better thermo-stable than the enzyme preparation without cofactor, indicating the stabilizing effect of cofactor against thermo-inactivation, as previously reported by Gupta, (1991) and Gestrelius, et al. (1975). In addition, the thermo-stability of the immobilized enzyme preparations with and without cofactor were better at lower RH (63.1 %) than at higher RH (74.5 %), which might be due to less water mediated thermo-inactivation of the immobilized enzyme at lower RH (63.1 %) (Turner, et al. 1995; Klibanov and Ahern, 1987 and Klibanov, 1986).

![Figure 4.34: Effect of cofactor-to-protein molar ratio on initial reaction rate and half-life of immobilized lyophilized LBADH (A) and immobilized lyophilized ADH T (B): (□) denotes initial reaction rate and (■) represents half-life. Gas-phase reaction conditions:

Flow rate 35 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 60, isopropanol activity 0.116, water activity 0.55, temperature 60 °C, 150 mg of immobilized enzyme preparation. Immobilization conditions: 170 units of lyophilized LBADH or lyophilized ADH T, 500 mg of non-porous plain glass beads, 1 ml of distilled water, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure.

The effect of the cofactor-to-protein (C/P) molar ratio on the initial reaction rate and the half-life of immobilized lyophilized LBADH and immobilized lyophilized ADH T was evaluated in the gas-phase reaction. The cofactor amount was varied while the protein amount was kept constant. It was found that the initial reaction rate and the half-life of the immobilized enzyme preparations increased almost linearly with the increase in C/P
molar ratio until a C/P ratio of 1.25 for LBADH and 1.78 for ADH T (Figures 4.34A and B). This might be attributed to an increase in number of enzyme-cofactor complexes leading to stabilization of the enzyme, as previously reported by Gupta, (1991) and Gestrelius, et al. (1975). With further increase in the C/P molar ratio, neither the initial reaction rate nor the half-life of immobilized enzyme preparations was significantly increased. This was perhaps due to the saturation of the enzyme active sites with the cofactor. Clearly, the C/P molar ratio of 1.25 for LBADH and 1.78 for ADH T was found as optimum. In general, the initial reaction rate of the immobilized ADH T was higher than the immobilized LBADH, as the enzyme in the former case was relatively more purified than the latter. Surprisingly, the thermo-stability of the mesophilic LBADH was higher than that of the thermophilic ADH T at all the C/P molar ratios (see Section 4.2.6.1).

Figure 4.35: Effect of cofactor on half-life of lyophilized LBADH and lyophilized ADH T enzyme in aqueous phase: White bars denote half-life without cofactor and black bars represent half-life with cofactor. Reaction conditions are described in Section 3.2.6.1.

In order to differentiate between the cofactor and enzyme thermo-inactivation, the thermo-stability of the lyophilized LBADH and lyophilized ADH T in aqueous phase was studied at 40 °C with and without addition of cofactor by measuring its residual activity periodically. As shown in Figure 4.35, the thermo-stability of both enzymes in aqueous phase with cofactor was better than without cofactor. This increase in thermo-stability might be due to the stabilizing effect of the cofactor binding the active site of

4.3.6 Effect of temperature

4.3.6.1 Initial reaction rate and half-life of immobilized enzyme preparation

Figure 4.36: Effect of temperature on initial reaction rate and half-life of immobilized lyophilized LBADH (A) and immobilized plain cell extract of ADH T (B): (□) denotes initial reaction rate and (■) represents half-life. Gas-phase reaction conditions: Flow rate 35 ml/min, acetophenone activity 0.15, isopropanol-to-acetophenone molar ratio 60, isopropanol activity 0.116, water activity 0.55, 150 mg of immobilized enzyme preparation. Immobilization conditions: 170 units of lyophilized LBADH and plain cell extract of ADH T, 500 mg of non-porous plain glass beads, 1 ml of distilled water for LBADH and 1 ml of 50 mM PO₄-buffer (pH 7) for ADH T, cofactor-to-protein molar ratio 1.25 for LBADH and 3.55 for ADH T, 4 °C temperature, 2 h stirring time, (125 ± 5) rpm stirring speed, 45 kPa absolute drying pressure, with sucrose: sucrose added 5 times the protein amount (w/w) to the plain cell extract of ADH T.

Temperature is one of the important parameters having a strong influence on the initial reaction rate and the thermo-stability of the immobilized enzyme preparation. The impact of the temperature was evaluated in the gas-phase reaction in the range from 40 °C to 70 °C keeping all other parameters constant. The results are shown in Figure 4.36. In case of immobilized LBADH and immobilized ADH T preparations, the initial reaction rate increased exponentially with the increase in temperature, while the half-life...
of the enzyme preparations followed exponential decay with the increase in temperature. The decrease in half-life was due to the deactivation of the enzyme, which was confirmed by measuring the residual activity of the enzyme after the completion of the reaction. The highest initial reaction rate of both the immobilized enzyme preparations was found at 70 °C, and the half-life was least also at 70 °C. Surprisingly, the thermo-stability of the mesophilic LBADH was higher as compared to the thermophilic ADH T except at 70 °C, where thermo-stability of both the enzymes was the same. In fact, the thermo-stability of both of the immobilized enzyme preparations was inversely related to the initial reaction rate. Similar observation with the lyophilized cells of *Rhodococcus erythropolis* was reported by Erable, et al. (2005).

Table 4.3: Comparison between LBADH and ADH T in terms of their gas-phase half-life and aqueous-phase half-life

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gas-phase half-life [h]</th>
<th>Aqueous-phase half-life [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBADH</td>
<td>990</td>
<td>4</td>
</tr>
<tr>
<td>ADH T</td>
<td>240</td>
<td>40</td>
</tr>
</tbody>
</table>

The reaction conditions in relation to Table 4.3 are described in Section 3.2.6.1.

The thermo-stability of LBADH and ADH T in the gas-phase was compared with that in aqueous phase at 40 °C (Table 4.3). Because the enzymes are known to be thermo-stable in dry form at low water activity (Volkin, et al. 1991; Barzana and Klibanov,1987; Klibanov, 1986 and Kimura, 1979), the thermo-stability of the enzymes in gas-phase was higher than that in aqueous phase. The gas-phase reactions are usually operated at controlled water content, while in aqueous phase the water-mediated thermo-inactivation of the enzyme results in drastic decrease in thermo-stability of the enzyme (Klibanov and Ahern, 1987 and Klibanov, 1986). Yang and Russell, (1996b) observed a similar effect of temperature on the thermo-stability of YADH in gas-phase and aqueous phase. Obviously, the thermo-stability of the thermophilic ADH T was found higher in aqueous phase as compared to that of the mesophilic LBADH. But this was reverse in gas-phase. The reason is still unknown.
4.3.6.2 Space-time yield and total turn over number of immobilized enzyme preparation

The volumetric productivity was expressed in terms of the space-time yield (STY) at the end of 80 h of the reaction. This is defined as the ratio of mass of product synthesized per reactor volume per unit time. The volume occupied by the immobilized enzyme preparation within the reactor column was referred as the reactor volume. For both the enzymes, taking their thermo-inactivation into consideration, the space-time yield of the chiral phenylethanol was calculated after 80 h from the start of the reaction. The efficiency of the catalyst was expressed in terms of total turn over number (TTN). Usually, for a cofactor-requiring enzyme, it is defined as the total number of moles of the product formed per mole of cofactor during the course of complete reaction (Chenault and Whitesides, 1987). But, due to the lack of possibility to measure the deactivation of the cofactor, the TTN was defined as the total number of moles of product formed per number of moles of catalyst consumed. The decrease in conversion observed during the gas-phase reaction might be attributed to the degradation of enzyme and/or the cofactor. As the degradation of enzyme could be measured, TTN with respect to enzyme was calculated. The STY of \((R)\)-phenylethanol production using LBADH and \((S)\)-phenylethanol using ADH T and the TTN of LBADH and ADH T were evaluated in the temperature range from 40 °C to 70 °C. As shown in Table 4.3 and Table 4.4, the STY increased with the increase in temperature until 60 °C and then it started decreasing. The decrease was perhaps due to the significant reduction in synthesis of the total amount of the product at the end of 80 h of the reaction, owing to thermo-inactivation of the enzyme. The maximum TTN of both the enzymes was achieved at 40 °C and then it decreased with the rise in temperature due to the drastic increase in thermo-inactivation of the enzyme. Both the STY and the TTN was higher for the mesophilic LBADH than for the thermophilic ADH T except at 70 °C where the thermophilic ADH T had both the STY and the TTN higher than mesophilic LBADH. The gas-phase reduction of prochiral ketones to chiral alcohols using S-ADH from *Thermoanaerobacter* species (ADH T) definitely showed higher STY (623 gm\(\cdot\)l\(^{-1}\)\cdot\)d\(^{-1}\)) than S-ADH from *Rhodococcus erythropolis* (READH) with the STY of about 63 gm\(\cdot\)l\(^{-1}\)\cdot\)d\(^{-1}\) (Kargl, et al. 1996) in aqueous phase. In addition, the STY of the chiral phenylethanol achieved in this study with both enzymes was higher than those obtained with the most successful industrial aqueous phase biotransformation using enzyme membrane reactor involving cofactor regeneration such as the synthesis of L-leucine.
(STY = 214 gm·l⁻¹·d⁻¹) and L-phenylalanine (STY = 366 gm·l⁻¹·d⁻¹) as reported by Kragl, et al. (1996).

Table 4.4: Effect of temperature on space-time yield (STY) and total turn over number (TTN) of immobilized lyophilized LBADH

<table>
<thead>
<tr>
<th>Temperature [°C]</th>
<th>STY [gm·l⁻¹·d⁻¹] at the end of 80 h</th>
<th>TTN</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>470</td>
<td>4 094 000</td>
</tr>
<tr>
<td>50</td>
<td>734</td>
<td>1 501 000</td>
</tr>
<tr>
<td>60</td>
<td>1098</td>
<td>919 000</td>
</tr>
<tr>
<td>70</td>
<td>398</td>
<td>194 000</td>
</tr>
</tbody>
</table>

Table 4.5: Effect of temperature on space-time yield (STY) and total turn over number (TTN) of immobilized plain cell extract of ADH T

<table>
<thead>
<tr>
<th>Temperature [°C]</th>
<th>STY [gm·l⁻¹·d⁻¹] at the end of 80 h</th>
<th>TTN</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>447</td>
<td>1 726 000</td>
</tr>
<tr>
<td>50</td>
<td>509</td>
<td>372 000</td>
</tr>
<tr>
<td>60</td>
<td>623</td>
<td>323 000</td>
</tr>
<tr>
<td>70</td>
<td>455</td>
<td>225 000</td>
</tr>
</tbody>
</table>
5 SUMMARY AND FUTURE WORK
This research work was aimed at development of a suitable immobilization and drying method for alcohol dehydrogenase (ADH) enzymes in order to obtain an efficient ADH enzyme preparation for production of enantiomerically pure alcohols with high space-time yield in gas-phase reaction. With this view, as a first step, effect of various immobilization (adsorptive) as well as drying process parameters on the immobilization efficiency was studied. The immobilization efficiency was expressed in terms of residual activity and protein loading. Optimizing various immobilization conditions, this research work registered about 80 %, 316 % and 325 % residual activity with YADH, LBADH, and ADH T, respectively. In general, it was observed that low temperatures and mild stirring conditions resulted in an increased residual activity. It was found that the phenomenon of bubble nucleation caused detrimental effects on the enzyme activity at low drying pressures. Besides this, a step-wise drying process was found more effective than a single step drying process. Moreover, addition of sucrose during the immobilization process increased the residual activity; but, on the other hand, decreased the protein loading. Additionally, the use of hydrophobic supports resulted in higher residual activity as compared to hydrophilic ones, but the protein loading was higher with the latter. Addition of a suitable buffer (50 mM phosphate buffer, pH 7) or an optimum amount of sucrose (5 times greater than the amount of protein on weight basis) during the immobilization enhanced the thermo-stability of the enzyme in gas-phase reaction.

Afterwards, the optimally prepared immobilized enzymes were used in gas-phase reaction with a view to optimize the gas-phase reaction parameters in order to achieve high productivity. It was found that the water activity increased the initial reaction rate, but, at the same time, decreased the thermo-stability (half-life) of the immobilized enzyme preparation. An optimum water activity was found as 0.55 for LBADH and ADH T. A critical cofactor-to-protein molar ratio was required to obtain high initial reaction rate and high thermo-stability of the immobilized enzyme preparation. This was found as 1.25 in case of LBADH and 1.78 for ADH T. A remarkable enhancement in the thermo-stability of the ADH enzymes was noticed. The space-time yield of (R)-phenylethanol was about 1000 gm·L⁻¹·d⁻¹ with LBADH and the space-time yield of (S)-phenylethanol was about 600 gm·L⁻¹·d⁻¹ with ADH T. The total turnover number of LBADH was about $9 \times 10^5$ and the same of ADH T was about $3 \times 10^5$. 

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It was interesting to notice that the thermo-stability of the thermo-stable ADH enzyme was higher than that of the mesophilic ADH enzyme in aqueous phase (conventional media), while in the gas-phase (non-conventional media) the thermo-stability of the latter was better than that of the former. Thus, this research work showed how to prepare efficient (high residual activity) immobilized ADH enzymes possessing high thermo-stability in gas-phase reaction and yielding high productivity (space-time yield and total turnover number). High enzyme activity of the enzyme preparation in the aqueous phase need not be an optimal enzyme preparation for the gas-phase reactions.

The empirical experiences gained from this research work can be useful for the practical application as stated earlier in this chapter; however, during the application one must take into account especially the properties of a buffer such as temperature dependency and hygroscopicity. In future, attempts can be made to optimize the immobilization and drying processes as well as gas-phase reaction with different new industrially relevant enzymes, supports, and substrates. This will probably lead to more generalize the relations among the immobilization and gas-phase reaction parameters and their mutual dependencies on immobilization efficiency, thermo-stability of the immobilized enzyme preparation, and productivity of gas-phase reactions.
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Biography


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APPENDIX
Appendix A  Detailed Drawing of Frings Turbine II\textsuperscript{nd}

(Here all the dimensions are mentioned as two times more than in the original.)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{frings_turbine_diagram}
\caption{Diagram of Frings Turbine II\textsuperscript{nd} showing Stator, Rotor, and 2 Magnets.}
\end{figure}
Figure A1: Detailed drawing of Fringes turbine II$^{nd}$
CURRICULUM VITAE
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EDUCATIONAL QUALIFICATIONS

1998–2000: Master of Science (Technology) in Bioprocess Technology, University Institute of Chemical Technology, India
1994–1998: Bachelor of Pharmacy, Principal K. M. Kundnani College of Pharmacy, University of Mumbai, India

ACADEMIC ACHIEVEMENTS

- Ranked Fifth in Bachelor of Pharmacy in University of Mumbai in 1998
- Qualified Graduate Aptitude Test in Engineering (GATE) on Pharmaceutical Science with 90.45 percentile score, conducted by Indian Institute of Technology Mumbai in 1998

FELLOWSHIPS

- Received “Deutsche Forschungsgemeinschaft Graduiertenkolleg - GK 440 Methoden in der Asymmetrischen Synthese” for carrying out doctoral research work in Germany during 2001–2004
- Received German Federal Research Ministry Scholarship for carrying out three-month research project on the topic ”Asymmetric Synthesis of Diols Using Epoxide hydrolases” in Forschungszentrum, Juelich (Germany), 2000
- Selected for Junior Research Fellowship at UICT, funded by University Grant Commission, Government of India during 1998–2000
- Received National Merit Scholarship, awarded by University of Mumbai in 1992

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- Trivedi, A., Spiessi, A., Dausmann, T., and Buechs, J., Enhancing the Thermostability of Immobilized ADH Enzyme for Gas-Phase Reactions
(Accepted for Oral Presentation), **Bioprocesses**, Wiesbaden, Germany, 10th–12th May 2005

- Spiess, A., Trivedi, A., Dausmann, T., and Buechs, J., Comparison of Immobilised Mesophilic and Thermophilic Alcohol Dehydrogenases for the Catalysis of Gaseous Substrates (Oral Presentation), *Biocatalysis Workshop on Biocatalysis in Non-Conventional Media*, Manchester, UK, 13th–14th April 2005

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- Buechs, J., Ferloni, C., Trivedi, A., Heinemann, M., Dausmann, T., and Hummel, W., Production of Optically Active Substances in Gas-phase with Macroscopically Dry and Therefore Very Stable Enzymes (Oral Presentation), *Bioprocesses*, Wiesbaden, Germany, 2004


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- Trivedi, A., Spiess, A., Daussmann, T., and Buechs, J., Study on Mesophilic and Thermophilic Alcohol Dehydrogenases (To be communicated to *Biotechnology Progress* very soon)