

Thermodynamic and Kinetic Phenomena in the Enzymatic Conversion of Acetophenone to 1-(*R*)-Phenylethanol in a Continuous Gas/Solid Reactor

Von der Fakultät für Maschinenwesen der Rheinisch-Westfälischen Technischen
Hochschule Aachen zur Erlangung des akademischen Grades einer Doktorin der
Ingenieurwissenschaften genehmigte Dissertation

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Tag der mündlichen Prüfung: 21. Dezember 2009

Diese Dissertation ist auf den Internetseiten der Hochschulbibliothek online verfügbar.

Acknowledgement

At this point, it is a great pleasure for me to thank all the people that made this thesis possible:

Prof. J. Büchs for entrusting me with a very interesting and challenging project as well as the great opportunity to work at his group.

Dr.-Ing. A. Spiess, my scientific advisor, for her encouragement, guidance and support, that enabled me develop a deep understanding of the topic and subsequently my own tools to proceed.

Priv.-Doz. Dr. M. Pohl, my second advisor, for her scientific input and fruitful cooperation within her group.

Prof. P. Jeschke for the supervision of my PhD exam.

The Deutsche Forschungsgemeinschaft (DFG) for financing this project within the BioNoCo Research Training Group.

S. Taubert and R. Petri for their technical support during the construction and operation of the gas/solid reactor set-up as well as U. Kosfeld and T. Heise for being always readily available to offer me not only their expertise but also their moral support.

All my BioNoCo colleagues, for sharing with me a productive 3-years time within BioNoCo. Above all, L. Kulishova for our nice collaboration.

All my BioVT colleagues, who created a nice atmosphere at work and offered me generously their cooperation and friendship. Among them, my beloved office-colleague and friend, M. Zavrel.

My students, for their valuable contribution to this project: C. Tagliabó, A. Funke, M. Jung, S. Wakimura, O. Diesendorf, M. Krähnke, J. McIntyre, M. Jordan.

All my friends in Aachen who made my days there brighter: Jannis, Ioanna, Georgia, Robert, Georgianna, Lila, Kostas, Alexander and the Georgiadis family.

Last but not least, I would like to thank my family, for their endless love and support at each and every step in my life...

Kurzfassung

Heutzutage setzen sich die Gas/Feststoff biokatalytische Systeme innerhalb des unkonventionellen Biokatalyse Bereichs besonders durch. Um die Gas/Feststoff Biokatalyse für analytischen oder synthetischen Zwecke zu nutzen, muss der jeweilige Prozess im Detail verstanden werden. Daher wurde der Aufbau und die Charakterisierung eines kontinuierlichen Gas/Feststoff Reaktors hier demonstriert. Die überstehenden thermodynamischen und kinetischen Phänomene, während der katalysierten Konversion von Acetophenon zu 1-(*R*)-Phenylethanol mit der gleichzeitigen Oxidation von 2-Propanol zu Aceton von Wildtyp LBADH, wurden beschrieben. Der Betrieb des Reaktoraufbaus wurde validiert und nachweislich die thermodynamische Steuerung erreicht.

Am validierten System wurde der Stoffübergang entlang dem Festbett untersucht und die axiale Dispersion der reagierenden Komponenten wurde vorausberechnet. Dennoch wurde die Dispersion über dem Festbett durch eine ausgeprägte (bis 6 mgAcetophenon/mgProtein) Acetophenon Adsorption überlagert.

Die Rolle des Wassers wurde durch Adsorptionsstudien erforscht. Die Hydratation des immobilisierten Enzyms wurde durch eine BET-Isotherme beschrieben. Es wurde gezeigt, dass das Präsenz von Saccharose im Enzympräparat sehr signifikant ist, insbesondere für Wasseraktivitäten höher als 0.5. Signifikante Hysteresis während der Wasserdesorption, bis 0.6 mgWasser/mgProtein für lyophilisiertes Enzym und bis 10 mgWasser/mgProtein für immobilisiertes Enzympräparat, wurde festgelegt. Die für die messbare Konversion am Reaktor minimal benötigte Wasseraktivität wurde zwischen 0.2 und 0.25 festgelegt. Dies entspricht ungefähr 5 mgWasser/mgProtein absorbiertes Wasser. Eine weitere Erhöhung der Wasseraktivität des Reaktionsgemisches führte zu einer fast exponentielle Zunahme der Konversion.

Eine kinetische Untersuchung wurde unter ausschließlich Reaktions-Limitierenden Bedingungen durchgeführt. Eine 1er-Ordnung Reaktion Kinetik bezüglich Acetophenon ($v_{max}/K_M=0.0046 \text{ } \mu\text{mol/min/IU}$) und eine Michaelis-Menten Kinetik bezüglich 2-Propanol ($v_{max}=0.0046 \text{ } \mu\text{mol/min/IU}$ und $K_M=0.105$) wurden nachgewiesen.

Die Stabilität der immobilisierten Wildtyp LBADH ohne Saccharose wurde unter normale Betriebsbedingungen nachgeforscht und die wichtige Rolle der Wasseraktivität wurde gezeigt. Der vor der Immobilisierung auftretende Aufreinigungszustand des Enzyms und das Handling des Enzympräparats wurden als entscheidende Faktoren für die Betriebsstabilität hervorgehoben. Eine Vergleichsstudie der Betriebsstabilität der Wildtyp LBADH und der G37D NADH-abhängigen Varianten zeigte, dass die Voraussage von Enzymeigenschaften der Daten in Flüssigkeit nicht zuverlässig ist.

Die Enantioselektivität des Enzyms im studierten Reaktionssystem wurde ebenso erforscht. Die Konversion von Acetophenon wurde mit hoher Enantioselektivität im Gas/Feststoff Reaktor durchgeführt und lieferte enantiomerische Überschusswerte von ungefähr 99.5 % bei allen verwendeten Betriebsbedingungen. Die Wasseraktivität des Reaktionsgemisches wurde als die maßgeblichste Einflussgröße festgestellt.

Während des Gesamtprojektes wurde sowohl die Leistung des Reaktors, als auch des immobilisierten Enzympräparats geprüft und in Frage gestellt. Folgende Schwächen der ausgewählten Immobilisierungsmethode wurden beobachtet: ausgeprägte diffusive Limitation bei hoher spezifischer Aktivität des immobilisierten Enzympräparats mit Saccharose, Auswaschung des Enzyms bei hoher Wasseraktivität, Einfluss des Handlings des Enzympräparats auf die Reproduzierbarkeit der Messungen usw. Zusammenfassend ist zu sagen, dass diese Effekte auf die Wahl hinweisen in der Zukunft stärkere, vermutlich kovalenten, Immobilisierungsmethode und porösen Immobilisierungsträger anzuwenden.

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Nomenclature

Roman Symbols

A	[-]	first Antoine coefficient
B	[K]	second Antoine coefficient
C	[K]	third Antoine coefficient
C	[mM]	concentration
d	[m]	diameter
D_h	[m]	vent diameter
D_{i,N_2}	[m ² /s]	vapor diffusivity
D_L	[m ² /s]	axial dispersion coefficient
E	[-]	enantioselectivity
E_0	[IU]	enzyme activity
ee	[-]	enantiomeric excess
f	[-]	dilution factor
k_{cat}	[s ⁻¹]	turnover number
k_d	[h ⁻¹]	deactivation constant
K_m	[mM] or [-]	Michaelis-Menten constant
m	[mg]	mass
M_0	[mg]	monolayer water coverage
p	[mbar]	partial pressure
P	[mM] or [mbar]	product concentration
P_{abs}	[bar]	absolute pressure
p^s	[mbar]	partial saturation pressure
Q	[μmol/min]	molar flow rate
R	[mL mbar/K/μmol]	gas constant
r	[m]	radius
S	[mM] or [mbar]	substrate concentration
T	[°C]	temperature
t	[s] or [h]	time
$t_{1/2}$	[h]	half life time

v	[$\mu\text{mol}/\text{min}/\text{IU}$]	specific reaction rate
V	[mL]	volume
$\dot{V}$	[mL/min]	volumetric flow rate
v_0	[$\mu\text{mol}/\text{min}/\text{IU}$]	initial specific reaction rate
v_{max}	[$\mu\text{mol}/\text{min}/\text{IU}$]	maximal specific reaction rate
z	[m]	axial dimension

Greek Symbols

α	[-]	thermodynamic activity
δ	[cm]	cuvette light path
ΔA	[-]	change in absorbance
ε	[-]	voidage
ε_{NADPH}	[mL/ $\mu\text{mol}/\text{cm}$]	extinction coefficient of NAD(P)H
μ	[kg/m/min]	dynamic viscosity
π	[-]	3.14
Π	[-]	degree of saturation
ρ	[kg/m ³]	density
σ	[kg/s ²]	surface tension
u	[m/s]	interstitial velocity

Subscripts

0	at zero time, initial
abs	absolute
i	component
L	axial
mug	make up gas
norm	normal
out	at the outlet
p	product
R	enantiomer R
S	enantiomer S
s	substrate
tot	total
w	water

Abbreviations

2-prop	2-propanol
Ac	acetone
AcPh	acetophenone
ADH	alcohol dehydrogenase
BET	Brunauer Emmett Teller
CSTR	continuous stirred tank reactor
FID	flame ionization detector
GC	gas chromatograph
HLADH	horse liver alcohol dehydrogenase
IU	international enzyme activity unit
LB	Lactobacillus brevis
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NAD(P)H	nicotinamide adenine dinucleotide phosphate
Pe	Peclet number
PhEtOH	phenylethanol
Re	Reynolds number
RH	relative humidity
TEA	triethanol amine
YADH	yeast alcohol dehydrogenase

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1. Introduction

1.1. Biocatalytic production of fine chemicals

Industrial biocatalysis has a long history, starting with the use of entire microorganisms. One of the oldest examples is the production of acetic acid from ethanol (known since 1815) with an immobilized *Acetobacter* strain. The racemic resolution of amino acids via the acylase method (in Tanabe, Japan, 1969) is one of the first industrial processes using isolated enzymes. Since then, more than 100 different biotransformations have been established in industry, mainly for the production of pharmaceuticals and agrochemical precursors, in most cases chiral compounds [Wandrey et al., 2000]. The development and application of efficient enzyme immobilization methods, at industrial scale, has been considered as the prerequisite for the industrial breakthrough.

Insoluble enzymes allow their application in continuous processes. There are today several examples of immobilized enzymes used in major commercial processes: production of glucose-fructose syrup using glucose isomerase (DSM, Degussa, Danisco/Genencor and others); production of 6-amino penicillanic acid using penicillin acylase (DSM, Pfizer and others); production of aspartame using thermolysin (DSM, Holland Sweetener Company); production of acrylamide and nicotinamide using nitrilase (Lonza); production of pharmaceutical and agrochemical intermediates using lipases (Novozyme, DSM, BASF, Fluka and others) [Poulsen, 1984; West, 1996; Cheetham, 2000; Liese, 2005; Schmid et al., 2000].

The asymmetric reduction of ketones using alcohol dehydrogenases as keto-reductases in particular is one of the most important ways to produce chiral alcohols, which can be transformed without racemization to industrially important chemicals, such as pharmaceuticals, agrochemicals and natural products [Nakamura et al., 2003].

1.2. Non-conventional biocatalysis

Traditionally, the sole medium suitable for enzymatic catalysis was considered to be a dilute aqueous solution. To date it is clear that both enzymes and whole cell biocatalysts can work far from their natural environment. Reaction media such as organic solvents, in one or two-phase systems [Halling, 1994; Vulfson et al., 2001], ionic liquids [Kragl et al., 2002; Park and Kazlauskas, 2003], supercritical fluids [Lozano et al., 2002; Lozano et al., 2004] as well as gases [Barzana et al., 1987; Lamare et al., 2004] are termed as non-conventional media and are currently employed for biocatalytic reactions.

1.3. Gas/solid biocatalytic reactions

1.3.1. Gas/solid biocatalytic reaction systems

The first studies devoted to the gas/solid catalysis date back 40 years and concerned an enzyme of which the natural substrate is gaseous hydrogen, the hydrogenase (hydrogen:ferricytochrome c₃ oxidoreductase, EC 1.12.2.1) from *Desulfovibrio desulfuricans* [Yagi et al., 1969]. It has been demonstrated that the hydrogenase in the dry state binds the hydrogen molecule and renders it activated resulting in para-hydrogen – ortho-hydrogen conversion, without the participation of aqueous protons in the reaction mechanism. Further studies [Kimura et al., 1979] have demonstrated that the enzyme in the dry state can also catalyze the reversible oxidoreduction of the electron carrier, cytochrome c₃ with hydrogen. The hydrogenase example is an example of enzyme acting on gaseous substrates offered by nature.

From the middle of the 1980s enzymes, that naturally convert dissolved substrates, were used in gas/solid reaction systems and the research on gas/solid biocatalysis was practically initiated. Since then, a number of gas/solid systems, employing either isolated enzymes or whole cells were studied and described.

Various enzyme classes and sub-classes, such as hydrolases [Parvaresh et al., 1992; Lamare and Legoy, 1995b/c; Barton et al., 1997; Lamare et al., 2001; Debeche et al., 2005; Graber et al., 2008], oxidoreductases like, alcohol dehydrogenases [Pulvin et al., 1988; Yang and Russell, 1996b; Ferloni et al., 2004] and alcohol oxidases [Barzana et al., 1987; Hwang et al., 1994a/b] even lyases [Mikolajek et al., 2007] have been employed in continuous or batch reaction systems, used either as enzyme powders [Parvaresh et al., 1992; Yang and Russell, 1996a/b] or being immobilized on various support types, porous [Pulvin et al., 1988; Indlekofer et al., 1996] or non porous carriers [Trivedi et al., 2005a/b] or even fibers [Debeche et al., 2005].

From the beginning of the 1990s whole cell gas/solid catalysis was introduced. Yeasts, like *Hansenula polymorpha* [Kim and Rhee, 2001] and *Saccharomyces cerevisiae* [Maugard et al., 2001] as well as bacteria like *Rhodococcus erythropolis* [Erable et al., 2005; Erable et al., 2004] and *Methylocystis sp.M* [Uchiyama et al., 1992] were employed in gas/solid reactors for oxidation, dehalogenation and hydrolytic reactions.

1.3.2. Advantages and limitations of gas/solid biocatalysis

Gas/solid biocatalysis presents several advantages when compared to more traditional biocatalytic systems (i.e. liquid aqueous, organic or biphasic systems). Its main strength is related to the increased stability of the enzymes and their cofactors in reaction systems with restricted water availability. The low water availability in these systems allows additionally performing reactions at increased temperatures, where higher reaction rates can be achieved and microbial contamination can be avoided. Additionally production of by-products is usually reduced or avoided. Moreover, substrate and product solubility problems are not

relevant for these systems and the use of harmful solvents can be avoided. The absence of solvents results in simplified downstream processing, which is usually performed through condensation and subsequent distillation [Parvaresh et al., 1992; Lamare et al., 2004; Debeche et al., 2005].

Nevertheless, the widespread application of the gas/solid systems remains limited compared to other systems. One major limitation is the requirement for volatile substrates and products. The elevated operational temperatures, usually employed in order to increase the substrates and products volatility and thus availability, create the need for thermostable enzymes. A solution to this problem may originate from the area of thermophiles and their thermostable enzymes. Previous studies [Trivedi et al., 2006] have indicated though, that the thermostability in the liquid phase is not necessarily transferred to the gas/solid systems, namely thermostable enzymes might be less stable when dried and used in a gas/solid system compared to their mesophilic counterparts. Moreover, the limited water availability in these systems is favorable, with respect to the enzyme stability but results in reduced enzyme reactivity. Finally, the gas/solid biocatalytic technology is a rather recent one and remains still widely unexplored.

1.3.3. Applications of gas/solid biocatalysis

Being a rather young technology, solid/gas biocatalysis enjoys today only limited application. The sole industrial application example is the production of aromas, through a gas/solid esterification of natural kosher alcohols and acids by the lipase Novozyme 435. A pilot plant has been developed and patented [Lamare and Legoy, 1999], reaching productivities of 1-1.5 kg_{ester}/h/kg_{catalyst} [Lamare et al., 2001b].

Nevertheless, a number of potential future applications of gas/solid biocatalysis have been proposed, such as its use for flavor aldehyde production [Pulvin et al., 1988], production of value-added chemicals from natural gases and biomass [Kim and Rhee, 2001], biotransformation of toxic volatile organic compounds (VOCs), like halogenated organic compounds [Erable et al., 2005a/b], biodegradation of

volatile trichloroethylene (TCE) [Uchiyama et al., 1992] or in general purification of gas streams and modification of compounds generated as vapors [Barzana et al., 1987] as well as gas phase biosensors construction [Barzana, 1995].

Especially in the case of alcohol dehydrogenases, which have been often used in aqueous solutions, their widespread application is limited, due to operational instability of the enzymes and their expensive cofactors, product inhibition, lack of stability of some substrates and products and the insolubility of most of them in aqueous solutions. The use of organic solvents to solubilize the substrates or products, on the other hand, presents different drawbacks, such as enzyme instability in organic solvents and low mass transfer rates to and within the enzyme particles. The aforementioned problems can be overcome by performing the respective reactions in a gas/solid system [Yang and Russell, 1996b].

Apart from the aforementioned potential applications of the gas/solid biocatalysis, it has been also proposed as a highly valuable research tool, mainly thanks to the ability to control and adjust individually the thermodynamic activities of the species in these systems [Lamare and Legoy, 1995b/c]. Indeed the most recent publications have been mainly focused on using the gas/solid systems as tools to retrieve information on the biocatalyst conformation and interaction with its environment in non-conventional media, rather than dealing with applied biocatalysis. Gas/solid systems have been, therefore, employed for the prediction of the intrinsic properties of the enzymes and the intrinsic effect of solvent [Graber et al., 2007; Graber et al., 2008] as well as the influence of water on biocatalysis [Graber et al., 2003b]. In the gas/solid system, the absence of solvents permits the study of enzyme kinetics when the microenvironment of the protein consists solely of the reaction substrates and products and water vapors. Thus gas/solid biocatalysis can be considered as an experimental tool that combined with molecular modeling can facilitate the elucidation of the structure-function relationships [Lamare et al., 1997].

1.4. Objective

In order to use the gas/solid system for analytical or synthetic purposes, the respective process has to be understood in detail. Therefore, the objective of the present work is to construct a continuous packed bed gas/solid enzymatic reactor and to describe it in terms of all occurring thermodynamic and kinetic phenomena, such as: gaseous reaction mixture equilibration, mass transfer along the packed bed, water adsorption, substrate and product adsorption, reaction kinetics, enzyme stability and catalyst enantioselectivity.

The studied reaction system is the enantioselective reduction of gaseous acetophenone to 1-(*R*)-phenylethanol, with concomitant oxidation of gaseous 2-propanol to acetone, catalyzed by dried and deposited onto non porous carriers *Lactobacillus brevis* alcohol dehydrogenase.

A detailed description of the constructed reactor is provided in the Materials and Methods chapter (section 3.4). The characterization of the reactor, including the validation and calibration of the overall set-up as well as the description of the saturation process, the flow pattern and dispersion of gaseous compounds over the packed bed, is performed and described (section 4.1). The characterized reactor is subsequently used for the investigation and quantification of the occurring phenomena at the studied reaction system. Therefore, the adsorption phenomena (section 4.2), the coupling of the enzyme hydration with the reaction (section 4.3), the enzymatic reaction kinetics (section 4.4) and deactivation kinetics (section 4.5) as well as the enantioselectivity (section 4.6) of the catalyst in the gas/solid system are studied and discussed. An effort to interconnect the separate phenomena, as well as to validate the employed immobilization method is made.

2. Theoretical Background

2.1. Continuous gas/solid reaction system

The gas/solid reactions require the presence of a gaseous phase (substrates and water vapors) and a solid phase catalyst (dried enzyme). The evaporated substrates and water are carried by means of an inert carrier gas (i.e. N_2) to the packed bed of immobilized enzyme. The substrates and water vapors are adsorbed to the dried enzyme, establishing gas/solid equilibrium and the reaction is performed. The reaction products are desorbed and carried away from the packed-bed by means of the carrier gas.

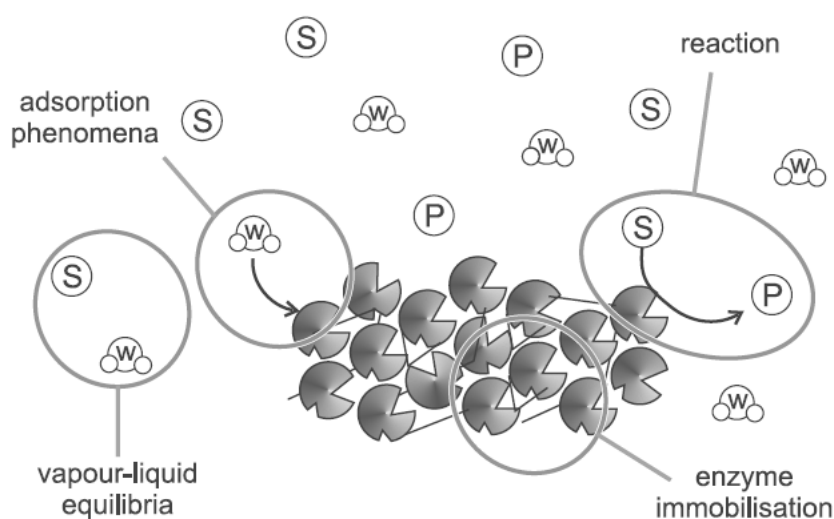


Figure 2.01 Gas/solid reactions principle [Ferloni, 2004]

The heterogeneous nature of these reactions results in an increased complexity, especially when they are performed in a continuous mode. Several overlaying

thermodynamic and kinetic phenomena take place in the gas/solid systems, including substrates and water distribution between the liquid and vapor phase, mass transfer, adsorption of substrates and water on the solid phase, reaction and subsequent desorption of the products. The principle of the gas/solid reactions is schematically presented in Fig.2.01.

2.1.1. Gaseous phase

The substrates and water participating in the gas/solid reactions are in the gaseous phase. The evaporation of these compounds is performed through the equilibration of the inert carrier gas with the respective pure liquids. At vapor-liquid equilibrium the amount of liquid taken up by the carrier gas corresponds to its saturation pressure at a constant temperature. In this way, the partial pressure of a substrate or water in the gaseous reaction mixture can be calculated.

The most commonly used vapor-liquid equilibrium equation giving the saturation partial pressure of a pure liquid p_i^s at a specific temperature is the semi-empirical Antoine equation (Eq.2.01) [Antoine, 1888], an equation derived from the Clausius-Clapeyron relation:

$$p_i^s = 10^{\left(A - \frac{B}{C+T}\right)} \quad \text{Eq. (2.01)}$$

where:

A	first Antoine coefficient	[-]
B	second Antoine coefficient	[K]
C	third Antoine coefficient	[K]
T	absolute temperature	[K]

The Antoine coefficients, A , B , C are compound-specific, refer to pure compounds and would differ for mixtures of different compounds. Their applicability is restricted to a specific temperature range. The Antoine coefficients for a number of

pure compounds, at defined temperature ranges can be found in various databases (DETERM, NIST).

In non-conventional biocatalysis, the availability of the substrates and water to the enzyme is not depicted through the partial pressure or the concentration of the respective compound, though. The relevant parameter is the thermodynamic activity. In the case of organic solvents, the calculation of the thermodynamic activities of the compounds requires the prior knowledge of the activity coefficients. On the contrary, for gas/solid systems, where the gas phase can be assumed as ideal, the thermodynamic activity α_i is calculated in a more straightforward manner, by the Eq. 2.02:

$$\alpha_i = \frac{p_i}{p_i^s} \quad \text{Eq. (2.02)}$$

where:

p_i partial pressure of the compound i [bar]

The partial pressures of the different compounds of the reaction mixture can be adjusted separately. Thus, independent control of the thermodynamic activity of each compound present in the microenvironment of the biocatalyst is achieved.

2.1.2. Solid phase

The solid phase in the gas/solid reactions is the enzyme itself. Although there are some mainly older studies, where lyophilized enzymes have been directly used in the gas/solid reactors [Parvaresh et al., 1992; Yang and Russell, 1996a/b], in most cases the enzymes are being used as immobilized catalysts.

The most obvious reason for immobilization is the need to reuse the enzymes, if they are expensive, in order to make their use in industrial processes economic [Buchholz et al., 2005]. The main advantage of immobilization is the facilitation of

continuous processes. Other advantages of enzyme immobilization but also some limitations posed by it are summarized in the following table:

Table 2.01 *Advantages and limitations of enzyme immobilization.*

Advantages	Limitations
Protection against inactivation by proteases / peptidases	Cost of carriers and immobilization
Stabilization of tertiary structure / restriction of unfolding	Possible activity loss during immobilization
Facilitation of product separation / recovery	Possible changes in enzyme properties
Co-factor stabilization through co-immobilization / recycling	Mass transfer limitations
	Potential problems with multi-enzyme systems

One additional difficulty is that a suitable immobilization method must be chosen according to the specific enzyme properties and be adjusted to the respective reaction system. The immobilization method development and validation is, therefore, one additional, often time consuming and costly step. A number of immobilization principles, for immobilization in carriers are presented in Fig.2.02.

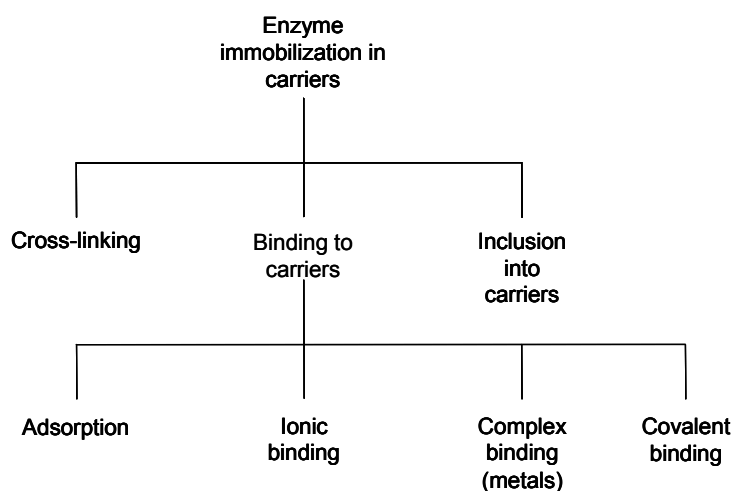


Figure 2.02 *Principles of enzyme immobilization in carriers [Buchholz et al., 2005].*

The classical principles for enzyme immobilization on carriers are physical adsorption, ionic binding to ion exchangers and covalent binding to an insoluble matrix with the two latter mostly used for technical applications [Buchholz et al., 2005]. Whereas covalent binding secures a strong attachment of the protein to the carriers it often results in unwanted irreversible binding, conformational changes of the enzyme tertiary structure and enzyme activity loss. Physical adsorption methods employ weaker interactions of the protein with the carrier (hydrophobic and van der Waals interactions) and as a result the enzyme activity and conformation are mostly preserved. Nevertheless, such methods are often not suitable for applications involving liquid media, due to the risk of enzyme leaching from the carriers [Sheldon, 2007].

On the contrary, in the gas/solid systems, enzyme leaching is normally avoided in the absence of liquid phase. Therefore, physical adsorption of enzyme on various material types is the most often selected immobilization method, as it is depicted in Table 2.02.

For the present project a simplified deposition process, similar to the one presented by Ferloni (2004) was selected for the immobilization of the LBADH onto non porous glass beads.

Table 2.02 Immobilized enzymes for gas/solid enzymatic reactions.

Enzyme	Immobilization method	Source
<i>C. antarctica</i> lipase B	adsorption onto Chromosorb P (mesh 60-80)	Graber et al., 2007
Horse liver and <i>S. solfataricus</i> alcohol dehydrogenase	cross-linking into albumin glutaraldehyde porous particles	Pulvin et al., 1988
<i>L. brevis</i> alcohol dehydrogenase	deposition onto non porous glass beads	Ferloni et al., 2004
<i>P. fluorescens</i> benzaldehyde lyase, <i>putida</i> benzoylformate decarboxylase	adsorption onto Chromosorb P (mesh 60-80)	Mikolajek et al., 2007
<i>F. solani pisi</i> cutinase	adsorption onto Chromosorb P (mesh 30-60)	Lamare et al., 1997
<i>P. cepaciae</i> lipase PS	adsorption onto glass fibers and carbon woven fabric	Debeche et al., 2005
Porcine lipase	adsorption onto glass wool	Barton et al., 1997
<i>M. miehei</i> lipozyme IM lipase	adsorption onto phenolic ion-exchange resin (Duolite 568)	Perez et al., 2007

2.2. Alcohol dehydrogenases

Alcohol dehydrogenases are abundant in nature and have been found in many microorganisms, plants and animal tissues. The typical reaction scheme they catalyze is depicted in Fig.2.03.

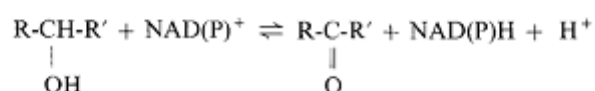


Figure 2.03 Alcohol dehydrogenase reaction scheme.

Depending on their biological source, they demonstrate different substrate specificities. Their preparative applications are limited due to their usual narrow substrate specificity. Two commercially available, rather inexpensive and often used alcohol dehydrogenases are isolated from yeast (YADH) and from horse liver (HLADH) [Hummel and Kula, 1989]. The alcohol dehydrogenases are either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) dependent.

NAD(P)-dependent alcohol dehydrogenases are useful catalysts for the synthesis of chiral compounds. Many active and stable enzymes are available that reduce pro-chiral ketones to chiral alcohols with high enantioselectivity. Due to the high cost of their cofactors though, a regeneration system is essential in preparative applications [Hummel, 1999]. Alternatively, dehydrogenases can also be applied in an oxidative manner to resolve the racemic mixture by oxidizing stereoselectively one enantiomer only. This route is advantageous if the corresponding ketones are difficult to prepare or the complementary enantiomer should be prepared [Hummel and Riebel, 2003].

2.2.1. *Lactobacillus brevis* alcohol dehydrogenase

The *Lactobacillus brevis* alcohol dehydrogenase (LBADH) used in this project is a member of the short-chain dehydrogenase/reductase (SDR) enzyme super-family. It is a homo-tetramer with 251 amino acid residues and a molecular mass of about 26.6 kDa per subunit and uses NADP(H) as co-enzyme. It was found in several *Lactobacillus* strains during a screen for novel biotechnologically interesting alcohol dehydrogenases. The enantio-specificity of the enzyme can be explained on the basis of the resulting hypothetical ternary complex. In contrast to most other SDR enzymes, the catalytic activity of LBADH depends strongly on the binding of Mg^{2+} . Mg^{2+} removal by EDTA inactivates the enzyme completely. In the crystal structure (Fig.2.04), the Mg^{2+} -binding site is well defined.

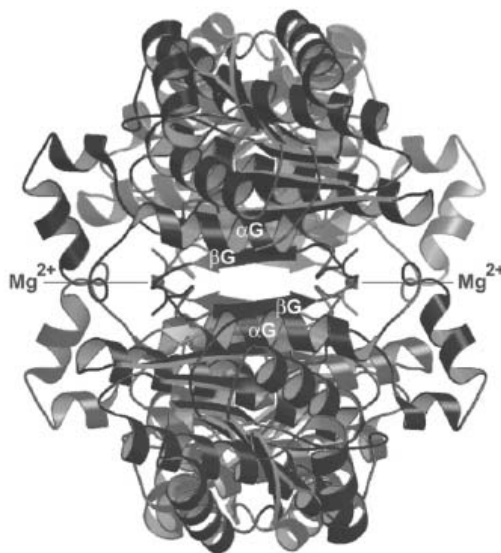


Figure 2.04 Quaternary structure of LBADH [Niefind et al., 2003].

The LBADH catalyzes reactions of the scheme of Fig.2.03. In this reaction scheme, R is typically a methyl group. In contrast, R' can vary over a wide range of residues and can be, in particular, a bulky moiety.

The preferred LBADH substrate for in vitro studies is acetophenone, which is not accepted as a substrate by any of the commercially available alcohol dehydrogenases [Hummel, 1997]. Catalyzed by LBADH, however, acetophenone is reduced to 1-(R)-phenylethanol with $\geq 99\%$ enantiomeric excess. The valuable

substrate and enantio-specificity of this enzyme make it an attractive catalyst for the production of chiral alcohols. [Niefind et al., 2003].

Nevertheless the requirement of the LBADH for NADPH (or NADP⁺) as coenzyme is a disadvantage for its use, due to the higher cost (factor 5-10) and reduced stability of this coenzyme, compared to NADH. For this reason a NADH-variant of this enzyme, G37D, was created through a single amino acid mutation (glycine replaced by aspartic acid) in the active centre reducing in this manner the alkalinity of the enzyme in the coenzyme docking area and aiming at increasing its NADH specificity [Riebel, 1997; Hummel and Riebel, 2002; Schlieben et al., 2005].

2.2.2. Continuous acetophenone reduction to 1-(*R*)-phenylethanol

The target reaction of the present project is the reduction of acetophenone to 1-(*R*)-phenylethanol by means of the LBADH, in a continuous mode. The essential co-factor, NAD(P)H, co-immobilized via deposition together with the enzyme on non-porous glass carriers, can not be stoichiometrically used, due to its high cost. A regeneration system is therefore required.

There are a few well established regeneration systems for this purpose [Wichmann and Vasic-Racki, 2005]. The first main approach, the so called enzyme-coupled regeneration system, involves a second enzyme, typically formate dehydrogenase, which catalyzes the oxidation of formate to CO₂ [Kruse et al., 1996]. The second approach uses the same enzyme that catalyzes a concomitant reaction, of an inexpensive substrate and is referred as substrate-coupled regeneration system. If the target reaction is a reduction, the parallel reaction is an oxidation and vice versa. There are finally some other ways of co-factor regeneration, like electrochemical methods or using H₂ with a hydrogenase [Findrik et al., 2005].

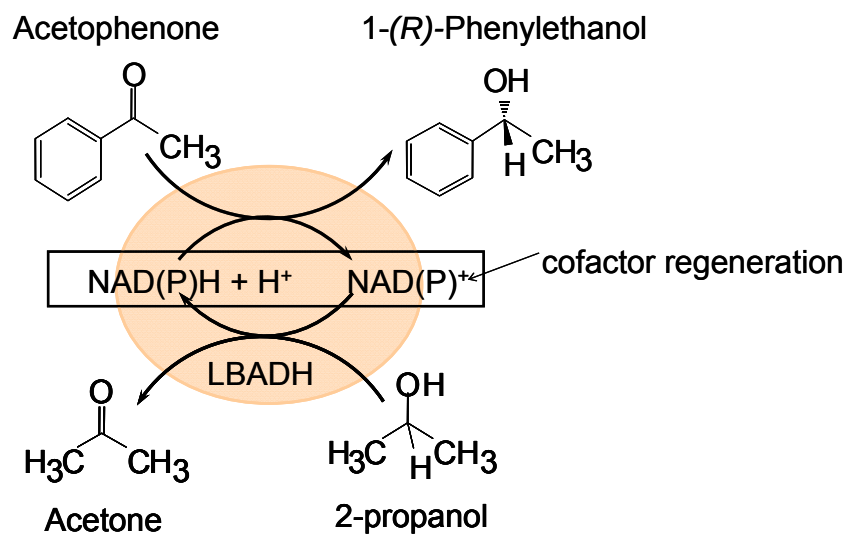


Figure 2.05 Reaction system for acetophenone reduction to 1-(R)-phenylethanol by means of immobilized LBADH with co-immobilized co-factor. The co-factor regeneration is performed through the substrate-coupled method.

The co-factor regeneration at this project is performed via the second method, namely the substrate-coupled regeneration system, as it is shown in Fig.2.05. 2-propanol is employed as second substrate and is oxidized by the LBADH to acetone, reducing in this way the oxidized cofactor (NAD(P)^+).

2.3. Reaction mechanism and kinetics

2.3.1. Reaction mechanism

The enzymatic reactions catalyzed by alcohol dehydrogenases involve two substrates, one of them being the oxidized or reduced cofactor and the second the organic compound (alcohol or ketone) to be converted. The cofactor binds first to the enzyme and then binds the organic compound. Theorell and Chance proposed a mechanism [Theorell and Chance, 1951] where no central complex is formed (Fig.2.06). Later it was found that a central complex is indeed formed at very low concentrations and, therefore, an ordered Bi-Bi mechanism can be assumed [Cleland, 1963a].

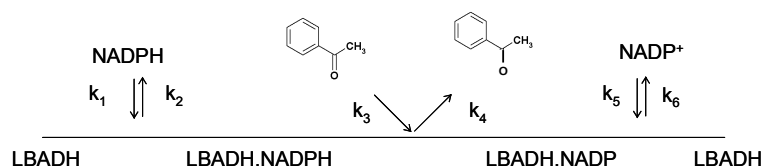


Figure 2.06 Acetophenone conversion by LBADH, following the Theorell and Chance mechanism.

In the case of the immobilized alcohol dehydrogenase with co-immobilized cofactor though, the mechanism should be simplified to a Uni-Uni mechanism, assuming that the cofactor is constantly fixed to the enzyme active center (Fig.2.07).

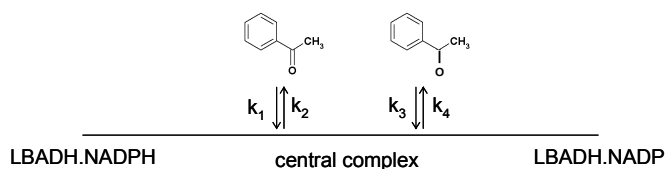


Figure 2.07 Acetophenone conversion by immobilized LBADH with co-immobilized cofactor, following a Uni-Uni mechanism.

Since two parallel reactions take place, utilizing the same enzyme, the substrates bind in an alternating fashion causing oxidation of the alcohols and reduction of the ketones.

2.3.2. Reaction kinetics: Michaelis-Menten

The kinetics of the enzyme catalyzed reactions can be described by the Michaelis-Menten equation, which gives the initial reaction rate as a function of the enzyme concentration under the assumptions that the total enzyme concentration is much lower than the substrate concentration and remains constant and that the substrate-enzyme complex formation and dissociation process is in steady state.

$$v_0 = v_{\max} \cdot \frac{S}{S + K_m} \quad \text{Eq. (2.03)}$$

where:

v_0	initial specific reaction rate	[$\mu\text{mol}/\text{min}/\text{IU}$]
$v_{\max}$	maximal specific reaction rate	[$\mu\text{mol}/\text{min}/\text{IU}$]
S	substrate concentration	[mM]
K_m	Michaelis-Menten constant	[mM]

The Michaelis-Menten constant (K_m) for a substrate of an enzyme depicts the substrate concentration at which the reaction rate reaches half of the maximum reaction rate. Consequently K_m is an indicator of the affinity of a specific substrate to the enzyme: the lowest the K_m value the highest the affinity of the specific substrate to the enzyme. At low substrate concentrations ($S \ll K_m$) the Michaelis-Menten equation is reduced to a first order kinetic:

$$v_0 \cong \frac{v_{\max}}{K_m} \cdot S \quad \text{Eq. (2.04)}$$

At high substrate concentrations ($S \gg K_m$), all active centers of the enzyme are saturated and therefore the maximal reaction rate is reached and the equation is reduced to a zeroth order kinetic:

$$v_0 \cong v_{\max} \quad \text{Eq. (2.05)}$$

2.3.3. Deactivation kinetics

Protein denaturation has been studied for many years. An effort has been made to determine the kinetic properties of an enzyme system undergoing the process of inactivation, and how these kinetic properties of the system may change under different environmental conditions. Besides, the study of enzyme inactivation provides an avenue to understand the structure-function relationships of enzymes [Sadana, 1988]. Since enzyme inactivation is one of the constraints in the rapid development of biotechnological processes, a better mechanistic understanding is required to facilitate and enhance the economic feasibility of enzyme-catalyzed processes [Sadana, 1988]. On the other hand, the thermal inactivation of enzymes and proteins has received much attention due to the great importance of thermal processes, like sterilization and pasteurization, in the food and pharmaceutical industry [Nath, 1995].

Enzymes tend to be highly defined structures, especially having compactly folded interiors. Although variations of the structure can occur, without destroying the catalytic ability of the enzyme, variation of the native form, e.g. substitution of amino acid residues, partial unfolding or dissociation, may affect its specific activity [Henley and Sadana, 1986]. Despite this complexity of the enzyme deactivation process, most studies propose a first-order deactivation kinetic, given by the following equation:

$$v = v_0 \cdot e^{(-k_d \cdot t)} \quad \text{Eq. (2.06)}$$

where:

k_d deactivation constant [1/h]

However, large deviations from first order kinetics have been previously reported for both immobilized and dissolved enzymes [Chang et al., 1988; De Cordt et al., 1992; Sadana, 1988]. These variations could originate from formation of enzyme groups with different stabilities or the presence of stable/labile isoenzymes or series-type enzyme inactivation kinetics [Sadana, 1988; Nath, 1995]. Previously

published examples of enzymes demonstrating a non-linear enzyme activity-time relationship are: the horseradish peroxidase [Chang et al, 1988] the *Bacillus licheniformis* α -amylase [De Cordt et al., 1994] and the baker's yeast alcohol dehydrogenase [Nath, 1995].

2.4. Enantioselectivity

Enantioselectivity is called the ability of enzymes to discriminate among enantiomers, enantiofaces or identical functional groups linked to a prochiral center. In the latter case it is also called prochiral selectivity [Carrea et al., 1995]. Enantiomers (or optical isomers) are stereoisomers that are non-super imposable complete mirror images of each other. The respective property of the compounds is called chirality and the compounds chiral. The chiral molecules are optically active, turning the plane of polarized light. A mixture of equal amounts of the two enantiomers is called racemic mixture and is optically inactive.

The designation of the two enantiomers of a chiral compound is performed in two main ways: according to the molecule configuration the enantiomers can be designated as *R*- or *S*-; depending on the direction in which they turn the polarized light, they are designated as (+)- or (-)-, for the compound turning the light clockwise or counterclockwise, respectively. The two aforementioned systems have no fixed relation.

The two enantiomers of chiral compounds may differ also in taste and smell but most importantly have different effects as drugs. Therefore, the stereoselectivity of the enzymes is increasingly applied to produce directly pure enantiomers from prochiral compounds (asymmetric synthesis) or racemic mixtures (racemate resolution) and avoid in this way the subsequent intricate, due to their high similarity, separation process of enantiomers.

The enantioselectivity E of an enzyme catalyzed reaction is defined as the ratio of the (*S*)- and (*R*)- enantiomer consumption or production rates (v_S and v_R):

$$E = \frac{V_S}{V_R} \quad \text{Eq. (2.07)}$$

For equilibrium controlled reactions, the enantioselectivity can be calculated through the ratio of the specificity constants of the two enantiomers, based on initial reaction rate measurements:

$$E_{eq} = \frac{(k_{cat}/K_m)_S}{(k_{cat}/K_m)_R} \quad \text{Eq. (2.08)}$$

A simpler but less accurate way to estimate the enantioselectivity is based on the determination of the enantiomeric excess % ee of the substrate or product and the extent of the reaction (conversion) when the enantiomeric excess is measured [Buchholz et al., 2005]:

$$\% ee_r(S) = \frac{S_R - S_S}{S_R + S_S} \cdot 100\% \quad \text{Eq. (2.09)}$$

$$\% ee_r(P) = \frac{P_R - P_S}{P_R + P_S} \cdot 100\% \quad \text{Eq. (2.10)}$$

R-alcohols with high enantiomeric excess can be obtained with the aid of the NADPH-dependent alcohol dehydrogenases from *Lactobacillus* strains. More specifically, $ee \geq 99\%$ has been reported for the production of 1-(R)-phenylethanol by the *Lactobacillus kefir* ADH [Hummel, 1997] using either the 2-propanol substrate-coupled regeneration system [Riebel, 1997] or the formate dehydrogenase enzyme-coupled regeneration system [Seelbach et al., 1996]. The *Lactobacillus brevis* ADH converts acetophenone to 1-(R)-phenylethanol also with $ee \geq 99\%$ [Niefind et al., 2003, Hummel, 1997], whereas covalently immobilized LBADH was reported to convert acetophenone to 1-(R)-phenylethanol with $ee > 99.5\%$ in a plug-flow reactor [Hildebrand and Lütz, 2006].

2.5. Tubular catalytic reactors

For biocatalytic applications, tubular reactors are applied mainly as fixed-bed reactors with immobilized biocatalysts (spheres or granules). The main advantage of fixed-bed reactors is the simple continuous operation. When compared to continuous stirred tank reactors (CSTRs), significantly higher catalyst productivity is obtained due to profiles of substrates and products inside the tubular reactor. Indeed in the fixed-bed reactors the substrates and products concentrations vary with the reaction time t or length z . Whereas in the case of CSTRs external mass transfer is normally not rate limiting, it can become rate limiting in fixed-bed reactors, depending mainly on the flow rate. Typical examples of application of tubular reactors are glucose isomerization and kinetic resolution of racemic amino acids [Buchholz et al., 2005].

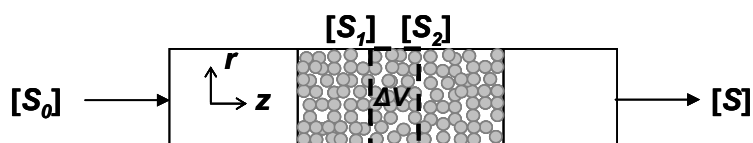


Figure 2.08 Fixed-bed reactor with ideal plug-flow.

In an ideal plug-flow reactor, no mixing is taking place on the axial direction, whereas there is perfect mixing in the radial direction. Thus, the substrates and products concentrations differ only on the axial direction. For a plug-flow reactor the mean residence time τ can be calculated by the following equation:

$$\tau = \frac{V}{\dot{V}} \quad \text{Eq. (2.11)}$$

where:

V	volume	[L]
$\dot{V}$	volumetric flow rate	[L/h]

Very often there is a deviation from the ideal plug-flow operation of a tubular reactor, depending on the hydrodynamics within the vessel. Dispersion along the path of the fluid may occur that can be attributed to turbulence, a non-uniform velocity profile, or diffusion.

Experimentally there are two ways to determine this deviation, namely through the step and pulse experiments. In a step experiment the concentration of tracer at the reactor inlet changes abruptly from 0 to C_0 . The concentration of tracer at the outlet C is measured and normalized to the concentration C_0 . The pulse experiment requires the introduction of a very small volume of concentrated tracer at the inlet of the reactor, such that it approaches the dirac delta function. Although an infinitely short injection cannot be produced, it can be made much smaller than the mean residence time of the vessel [Levenspiel, 1996].

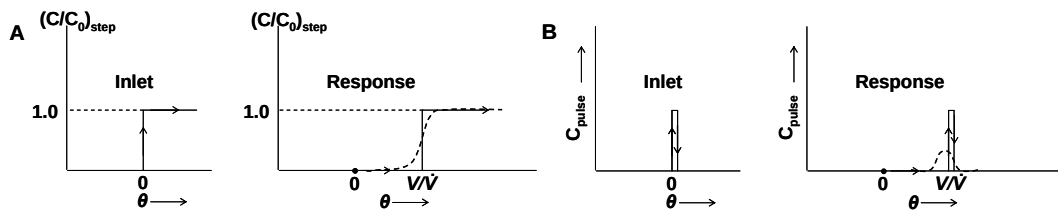


Figure 2.09 Performance in a tubular reactor: (A) step experiment and (B) pulse experiment. The straight lines represent the response in an ideal plug-flow reactor, while the dashed lines give a possible profile of a real reactor.

In a step experiment, the imposed concentration C_0 of the tracer, at the time point zero will start exiting the reactor at time equal to the residence time τ (Fig.2.09A), if the reactor behaves ideally, whereas the deviation from ideality will lead to a profile like the one indicated in the same figure by the dashed line. Similarly in a pulse experiment, the pulse will exit the reactor at time equal to the residence time τ , if the reactor behaves ideally, whereas for a reactor deviating from ideality, a residence time distribution (dashed line) will be observed (Fig.2.09B).

The flow in a real tubular fixed-bed reactor can be simulated by the flow in a tubular reactor, where the axial (z-direction) dispersion is proportional to the dispersion coefficient [Smith, 1981].

$$\frac{\partial C_i(z)}{\partial t} = -\frac{\dot{V}}{\pi \cdot r^2 \cdot (1 - \varepsilon)} \cdot \frac{\partial C_i(z)}{\partial z} + D_L \cdot \frac{\partial^2 C_i(z)}{\partial z^2} \quad \textbf{Eq. (2.12)}$$

where:

r	reactor radius	[m]
ε	voidage	[-]
z	axial direction	[m]
D_L	axial dispersion coefficient	[m ² /s]

2.6. The role of water in the gas/solid biocatalysis

The role of water during biocatalysis is multifaceted. Water is a substrate of a hydrolytic reaction or the product of the respective synthetic reaction. For any reaction, water can act as solvent facilitating the diffusion of the reactants. Water is required to form and maintain the native catalytically active conformation of enzyme molecules [Rupley and Careri, 1991]. On the other hand most reactions resulting in enzyme deactivation, mainly thermo-inactivation, require water [Barzana et al., 1989].

With respect to biocatalytic systems with low water content, many studies have been performed, aiming at understanding the role of water on the catalytic activity of the partly dehydrated enzymes [Zaks and Klibanov, 1985; Klibanov, 1987; Bell et al., 1995; Halling 1994; Halling 2004; Dunn and Daniel, 2004]. It has been shown that enzymatic activity is possible at very low hydration [Valivety et al., 1992]. For those systems, it has been demonstrated that the catalytic activity is determined by the water bound to the enzyme, rather than the total water content of the system [Zaks and Klibanov, 1988].

In non-conventional biocatalysis, it has been suggested that the thermodynamic state of water, described by the thermodynamic activity (α_i), should be taken into account in order to rationalize the observed effects [Drapon, 1985]. The water activity determines the mass action effects of water on hydrolytic equilibria and describes the distribution of water between the various phases that can compete in binding water [Halling, 1994]. Water activity has been considered as the key parameter for non-conventional biocatalysis. Whereas in the case of most non-conventional media, e.g. liquid organic media, the determination of the thermodynamic activity is complex, requiring the knowledge of activity coefficients, in the case of gas/solid catalysis the determination is much more straightforward [Lamare et al., 2004].

The water thermodynamic activity α_w in the gas/solid systems is determined through the ratio of the partial pressure of pure water over the partial saturation pressure of water at a specific temperature and it is related to the relative humidity of the system by the following equation:

$$\alpha_w = \frac{p_w}{p_w^s} = \frac{\%RH}{100} \quad \text{Eq. (2.13)}$$

where:

p_w	partial pressure of water	[bar]
p_w^s	saturation partial pressure of water	[bar]
$\%RH$	relative humidity	[-]

Hydration of dried enzymes is the incremental addition of water until a dilute solution is obtained. At some level of hydration there is sufficient water to completely saturate the molecule; the additional water only dilutes the system [Rupley and Careri, 1983]. A tool to describe the hydration of proteins [Dunn and Daniel, 2004] and the state of water on the microenvironment of the biocatalyst in systems of low water content is the water adsorption isotherm [Drapon, 1985].

A typical water adsorption isotherm (Fig.2.10) shows two characteristic break points, A and B. Point A corresponds to the water activity level below which the water is highly structured and forms the first hydration layer (water monolayer) of the dried enzyme. The intercept with the y-axis at $\alpha_w=0$ corresponds to the tightly-bound water amount. Between points A and B, the water content changes in a linear fashion with increasing α_w , resulting in the formation of subsequent hydration layers. Point B denotes the water activity level above which free water appears [Drapon, 1985].

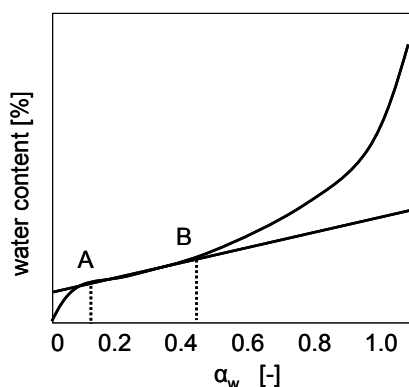


Figure 2.10 Water adsorption isotherm [4].

Although the water adsorption isotherm has the same general shape for almost all proteins (Fig.2.10), different proteins have somewhat different degree of hydration (different amount of adsorbed water) when equilibrated at the same water activity [Dunn and Daniel, 2004].

In gas/solid catalysis in particular, the role of water activity on the stability and activity of the dried enzyme has been the central topic of many studies. The minimal water activity required for the dried enzyme to become active has been defined for various gas/solid reaction systems [Barzana et al., 1989; Trivedi et al., 2006] and efforts have been made in correlating the hydration of protein, at this minimum water activity level, to the protein surface coverage with water molecules [Yang and Russell, 1996a]. The enhanced thermo-resistance of dried enzymes compared to enzymes dissolved in aqueous solutions has been proven [Barzana et al., 1987]. It has been also demonstrated that the role of water in gas/solid reactions is contradictory, namely increased water activity has a positive effect on the initial enzyme activity but a negative one on the enzyme stability. This dual water role has been demonstrated for trans-esterification reactions where the effect of water activity on the enzyme activity and stability was additionally correlated to the state of water in the system, deduced by the water adsorption isotherm of the dried enzyme [Robert et al., 1992]. The optimal water activity with respect to productivity was also defined for alcohol dehydrogenases catalyzing the acetophenone reduction to 1-(*R*)-phenylethanol [Trivedi et al., 2006]. The effect of water activity on the catalytic action of alcohol oxidase on ethanol vapors has also

been studied, indicating a 10^4 -fold increase of enzyme activity as the water activity increased from 0.11 to 0.84 [Barzana et al., 1989].

There are numerous further examples of studied enzymatic gas/solid reactions aiming at investigating the water influence on their performance [Lamare and Legoy, 1995; Parvaresh et al., 1992; Cameron et al., 2002; Bousquet-Dubouch et al., 2001], whereas more recent studies have used the gas/solid reactors as tools to elucidate the role of water in non conventional biocatalysis [Graber et al., 2003a/b].

3. Materials and Methods

3.1. Materials

3.1.1. Enzymes

The main part of the experimental results were acquired using purified and lyophilized wild type LBADH expressed in *E. coli*, obtained from Liliya Kulishova, Institute of Molecular Enzyme Technology (IMET) at Research Center Jülich, according to B. Riebel, 1996 (PhD thesis, Heinrich-Heine Universität Düsseldorf, Germany). The resulting lyophilized enzyme had a concentration of 0.4 mg_{protein}/mg_{solid}, the rest being salts resulting from the buffers (10 mM triethanol amine buffer (TEA) and 1mM MgCl₂) used during the purification process, and a specific activity of 88 IU/mg_{protein}. The lyophilized enzyme was stored at -20°C, in order to retain its initial activity.

Enzyme hydration and enantioselectivity studies were carried out using cell extract of wild type LBADH, expressed in *E. coli*, obtained by Jülich Chiral Solutions GmbH (Jülich, Germany). The cell extract had 3850 U/mL initial activity of LBADH and 28.6 mg/mL total protein concentration. The cell extract was stored at 8°C and was regularly tested before use in order to follow potential reduction of its activity during storage.

Finally, stability studies were carried out using deposited purified solution of the NADH dependent LBADH variant, G37D, expressed in *E. coli*, as well as deposited purified and lyophilized enzyme obtained also from Liliya Kulishova. The enzyme in solution had a concentration of 7 mg/mL and a specific activity of 7.14 IU/mg, while the lyophilized enzyme a concentration of 0.233 mg/mg and a specific activity of 3.67 IU/mg_{protein}. The solution was separated in aliquots which were kept frozen at -20°C and was also regularly tested to ensure the retention of its initial activity.

3.1.2. Chemicals

Acetophenone and 2-propanol were of >98% purity and purchased from Sigma-Aldrich (Buchs, Switzerland). All other chemicals, triethanolamine hydrochloride, Na₂HPO₄ and KH₂PO₄, used for the TEA and phosphate buffers, respectively, were of analytical grade and obtained from Roth (Karlsruhe, Germany). Bradford reagent and BSA were obtained from Bio-Rad (CA, USA).

3.1.3. Co-factors

The essential cofactors, NADP⁺ and NAD⁺, used for the co-immobilization with the wild type and mutant LBADH respectively, as well as their reduced forms, namely NADPH and NADH, used for the activity assays of the two enzymes, were obtained by Biomol (Hamburg, Germany).

3.2. Enzyme characterization

3.2.1. Protein concentration (Bradford)

Bradford test [Bradford, 1976] was applied for the determination of protein concentration in solution. For calibration, different dilutions of a BSA stock solution in H₂O with concentrations of 1 µg/mL, 5 µg/mL, 10 µg/mL, 12.5 µg/mL and 25 µg/mL were prepared. 800 µL BSA assay buffer of each dilution mixed with 200 µL Bradford reagent was introduced to cuvettes. Absorbance at 595 nm was measured by means of a UVIKON 922 photometer (Kontron Instruments, UK) after incubation for 5 min. The absorbance was plotted as a function of the protein amount giving the calibration curve. The unknown protein concentration was determined by adding 200 µL Bradford reagent to 800 µL of diluted protein solution. After 5 min the absorbance was measured at 595 nm. The protein amount in solution could then be determined from the calibration curve.

3.2.2. Enzyme activity

The determination of wild type (or mutant) LBADH activity was based on the measurement of the decrease in extinction at 340 nm, occurring when NADPH (or NADH) is oxidized to NADP⁺ (or NAD⁺) during the enzymatic reduction of acetophenone to phenylethanol. 970 µL of standard substrate solution (11 mM acetophenone and 1 mM MgCl₂ in 50 mM TEA buffer of pH 7) and 20 µL of cofactor solution (9.5 mM β-NADPH or NADH in 50 mM TEA buffer of pH 7) were pipetted into a 1 mL plastic cuvette and introduced into the thermostated UVIKON 922 photometer measuring chamber, where the solution was incubated for 5 min at 30°C. Subsequently 10 µL of appropriately diluted enzyme solution (so that the final activity would be in the range of 0.4 to 1.5 IU/min) were added, shortly mixed and measured directly after. The extinction decrease was measured for 1 min with a measurement frequency of 50 min⁻¹.

The enzymatic activity expressed as international units (IU) per milliliter was calculated using the following equation:

$$\text{IU/mL} = \frac{\Delta A/\text{min} \cdot V_{\text{cuvette}} \cdot f_{\text{dilution}}}{\epsilon_{\text{NADPH}} \cdot \delta \cdot V_{\text{enzyme}}} \quad \text{Eq. (3.01)}$$

where:

$\Delta A/\text{min}$	Change of absorbance	$[\text{min}^{-1}]$
V_{cuvette}	Volume of the assay	$[\text{mL}]$
f_{dilution}	Dilution factor	$[-]$
ϵ_{NADPH}	Extinction factor of NADPH or NADH	$[6.22 \text{ mL}/\mu\text{mol}/\text{cm}]$
δ	Light path of the cuvette	$[\text{cm}]$
V_{enzyme}	Volume of the pipetted enzyme solution	$[\text{mL}]$

3.3. Enzyme preparation and characterization for the gas/solid reactor

3.3.1. Enzyme immobilization via deposition

Applying a variation of the deposition procedure described in [Trivedi et al., 2005], an appropriate amount of IU of lyophilized enzyme, sucrose (5 times enzyme mass), when used, and NADP^+ (or NAD^+) (12 times molar enzyme amount), were dissolved in 1 mL phosphate buffer ($I=50 \text{ mM}$ and $\text{pH } 7$, resulting from KH_2PO_4 and Na_2HPO_4 solutions) and mixed together with 500 mg washed with distilled water and dried glass beads 0.25-0.3 mm (Sartorius, Germany) for 30 minutes in a rotary mixer (RMSW, Welabo, Germany) at 4°C . The mixture was dried at 4°C in a desiccator, containing Silica Gel Orange 2-5 mm (Carl Roth GmbH, Karlsruhe, Germany), with a vacuum pump (CVC 200II, Vacuubrand, Germany) at 300 mbar for 4 hours and successively at 40 mbar until dry. The enzyme preparations were stored at 4°C .

3.3.2. Enzyme residual activity

For measuring the residual activity of the deposited enzyme preparations, the deposited enzyme was dissolved from the carriers' surface by means of an appropriate amount of TEA buffer and the activity of the resulting solution was measured as previously described. The activity of the deposited enzyme in IU/mg_{carriers} was calculated taking into account the amount of carriers in mg dissolved in the TEA buffer amount in mL used.

3.3.3. Protein loading (Bonde)

In order to determine the amount of protein immobilized on the carriers a Bonde test was carried out [Bonde et al., 1992]. An amount of 10 mg of beads with immobilized enzyme were weighted into a cuvette and 800 µL TEA buffer as well as 200 µL Bradford reagent were added to the beads and mixed thoroughly. After incubation of 5 min and settling of the beads, the absorbance of the solution was measured at 465 nm by means of a UV-VIS spectrophotometer (UNIKON 922, Kontron Instruments, Italy). The protein amount immobilized on the beads was calculated using a BSA calibration curve.

3.3.4. Water content (Karl-Fischer titration)

The water content of the deposited enzyme preparations was determined by means of the volumetric Karl-Fischer titration. The flasks and lines of the titration stand (Schott Titration Stand TM KF, Schott AG, Mainz, Germany) and the automatic titrator (Schott Titroline alpha, Schott AG, Mainz, Germany) were dried prior to assembly: the lines were rinsed with acetone and finally blown through with compressed air and the reservoir bottles as well as the reaction vessel were dried in a cabinet drier for one day. The reservoir bottle of the automatic titrator was filled with Karl-Fischer reagent (Hydranal Composite 5) and the reservoir bottle of the titration stand with catalyst solution (Hydranal Methanol Rapid). Karl-Fischer reagent was directed through the lines of the titrator until all air bubbles

were removed. Subsequently, the reaction vessel was filled with catalyst solution until the electrode tips were below the liquid surface. To remove remaining water from the solvent, the reaction vessel was conditioned before any titration step.

To measure the water equivalent, eight fractions of circa 0.5 g water standard were applied to the vessel using a syringe. After each application, KF reagent was titrated on until a final value of 10 μ A of current was reached. The on average titrated volume was used to calculate the water equivalent:

$$Eq = \frac{m_s \cdot W_s}{V_{KF}} \quad Eq. (3.02)$$

where:

Eq	water equivalent of KF reagent	[mg/mL]
m_s	weight of analyzed Hydranal Standard	[g]
W_s	water content of the Hydranal Standard (= 10.05)	[mg/g]
V_{KF}	volume of consumed KF reagent	[mL]

The titration procedure was repeated with the enzyme preparation in the same manner as described above. For each preparation two fractions of 200-330 mg were applied and finally, the water content was calculated from the averaged volume of titrated KF reagent:

$$W_{enzyme} = \frac{V_{KF} \cdot Eq}{m_{enzyme} \cdot 10} \quad Eq. (3.03)$$

where:

W_{enzyme}	water content of the enzyme preparation	[%]
m_{enzyme}	weight of the applied enzyme preparation	[mg]

3.4. Enzymatic gas/solid reactions

3.4.1. Continuous gas/solid reactor

A new continuous gas/solid enzymatic reactor was constructed according to [Lamare and Legoy, 1995] for the needs of the present project and is schematically presented in Fig.3.01. The constructed gas/solid reactor was characterized and subsequently employed for the substrate adsorption, kinetic and stability studies.

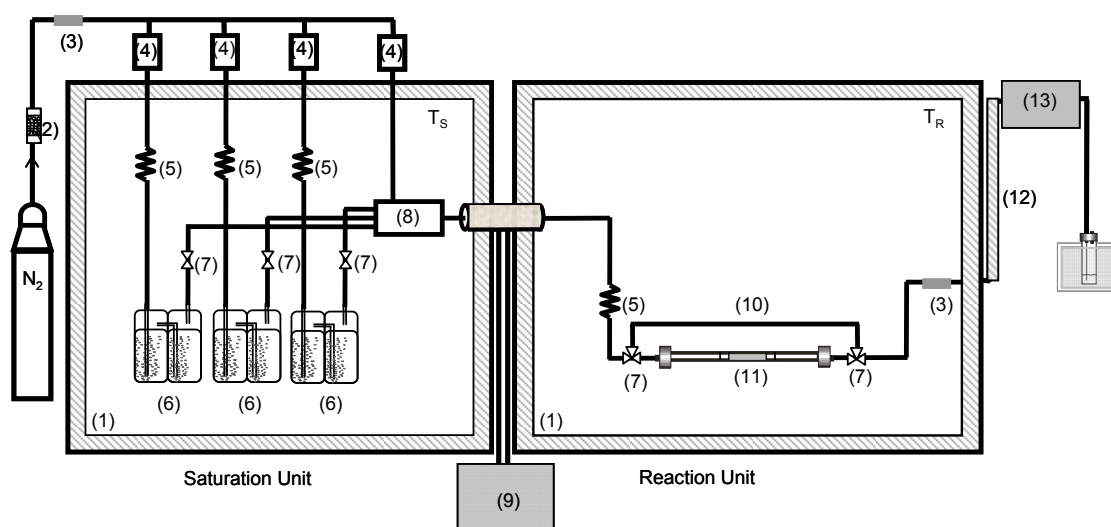


Figure 3.01 Continuous gas/solid enzymatic reactor: (1) thermoconstant chambers, (2) humidity trap, (3) filter, (4) mass flow controller, (5) heating coil, (6) substrate flask, (7) valve, (8) mixing chamber, (9) water-bath, (10) reactor by-pass, (11) packed bed reactor, (12) heating hose, (13) online GC.

The reactor consists of two separate, thermostated units: the saturation and the reaction unit. The two units can be in this way kept at different temperatures, by means of two thermoconstant chambers, BD53 and KB53 (Binder GmbH, Tuttlingen, Germany).

Nitrogen as carrier gas was dried, by means of a humidity trap (MT200-2, Agilent Technologies, Stuttgart, Germany) and rendered particle-free, by means of a Swagelok filter SS-2F-15 (B.E.S.T. Fluidsysteme GmbH, Düsseldorf, Germany). It

was directed through stainless steel tubing, of 2 mm inner diameter and approximately 5 m length (CS Chromatographie, Service GmbH, Langerwehe, Germany) and entered each saturation flask and while bubbling in the various substrates (acetophenone, 2-propanol and water) became saturated with them.

The saturation flasks were two-compartment glass or stainless steel flasks, favoring higher gas-liquid contact times and thus complete saturation of the carrier gas with the respective compounds. The flow rates of the carrier gas entering the three flasks were regulated by means of three mass flow controllers, of the type F-200CV-AAD-11-V and F-201CV-AAD-11-V (Bronkhorst Mättig GmbH, Kamen, Germany). A fourth mass flow controller of the type F-201CV-AAD-11-V regulated the flow of a nitrogen stream, referred as make-up gas.

Three 2-way valves (6604, Bürkert, GmbH, Germany) were positioned at the outlet of the substrates flasks in order to avoid back-mixing. The outlet streams of the saturation flasks, containing the carrier gas saturated with vapors of each component were mixed with the make-up gas stream in a cylindrical stainless steel tube (5.4 cm and 5 cm diameter and length respectively), serving as the mixing chamber.

The resulting mixture was subsequently directed through an externally thermostated, by means of a water bath, stainless steel pipe to the reaction unit. In order for the gas mixture to acquire the reaction unit temperature a stainless steel coil (5 m length) was used. The mixture was then directed, by means of two 2-3-way valves (6604, Bürkert, GmbH, Germany) either through the packed-bed reactor or its by-pass. The packed bed reactor consisted of a glass tube (5 mm inner diameter and 20 cm length) filled with 0.25-0.3 mm diameter glass carriers (Sartorius, Göttingen, Germany), retained with two glass wool layers on each side. The gas mixture was directed from the reactor outlet through a stainless steel filter (Swagelok, B.E.S.T. Fluidsysteme GmbH, Düsseldorf, Germany) to an online gas chromatograph. The inlet to the online GC valve was preheated to 100°C by means of a heating hose ELH/aiw-200, equipped with a temperature regulation system ELTC (Eltherm Elektrowärmetechnik GmbH, Burbach, Germany).

The operation of the gas/solid reactor was automated. The setting of the mass flow controllers and the switching of the valves was performed via a LabView (National Instruments, Texas, USA) program, created at the Chair of Biochemical Engineering, RWTH, Aachen.

The enantioselectivity studies were exclusively performed in the prototype III reactor designed by [Ferloni, 2005] and described elsewhere [Mikolajek, 2008]. The reaction progress was monitored online, by means of an online gas chromatograph. The enantioselectivity data were obtained through offline chromatographic measurement of samples collected at the outlet of the reactor, throughout each experiment.

3.4.2. Online gas chromatography

The reaction progress in the newly constructed gas/solid reactor was monitored by means of an online gas chromatograph (FISONS GC 8000, S+H Analytik, Mönchengladbach, Germany). Sampling was performed via a six-way Valco valve (VICI AG International, Schenkon, Switzerland) maintained at 150°C. The gas chromatograph was equipped with an FID detector, maintained at 250°C and a split/split-less injector, maintained at 200°C. The carrier gas was nitrogen with a head pressure of 260 kPa. Hydrogen and air were supplied at a head pressure of 50 kPa and 120 kPa, respectively. The separation was realized through a CP-WAX 52CB (50 m x 0.25 mm x 0.2 µm) GC column (Agilent Technologies, Stuttgart, Germany) at the temperature program presented in Table 3.01.

Table 3.01 Gas chromatography program for the online FISONS GC.

Rate [°C/min]	T [°C]	Time [min]
-	60	2
40	100	0
60	220	3

The analysis duration was 8 min and the time required by the GC to cool down to the initial temperature was approximately 7 min. As a result the sampling frequency was approximately $1/15 \text{ min}^{-1}$.

The retention times of the reaction mixture components are presented in Table 3.02 and an exemplary chromatogram is included in Appendix A. The analysis of the results as well as the operation of the 6-way valve was performed with the Chrom-Card 98 V.1.16 (FISONS) software.

Table 3.02 *Retention time of the reaction components for the online GC analysis.*

Component	Retention time
Acetone	2.64 min
2-propanol	2.98 min
Acetophenone	6.98 min
1-Phenylethanol	7.53 min

The calibration of the GC for the reactants and products was performed online with gas mixtures of known partial pressures for these compounds, created in the reactor saturation unit. The calibration of the GC for 1-phenylethanol in particular was also performed by equilibrating a nitrogen flow through a flask containing this compound situated in the reaction unit, directly before the exit of the reactor to the GC, in order to avoid the long-lasting adsorption of this compound onto the inner surface of the reactor set-up tubing. The online operation of the GC led to slight changes of the GC column which created the need for regular recalibration. One set of calibration curves for all compounds is included in Appendix A.

The reaction progress at the second reactor (prototype III), used for the enantioselectivity studies was monitored by a Hewlett, Packard 5890A (Santa Clara, USA) online gas chromatograph, equipped with a six-way Valco valve (VICI AG International, Schenkon, Switzerland), maintained at 175°C . The gas chromatograph was equipped with an FID detector, maintained at 250°C and a split/split-less injector, maintained at 180°C . The carrier gas was helium with a head pressure of 150 kPa. The separation was realized through a

CP-Chirasil-DEX CB (25 m x 0.25 mm x 0.25 μ m) GC column (Varian, USA) at the temperature program presented in Table 3.03.

Table 3.03 Gas chromatography program for the online HP GC.

Rate [°C/min]	T [°C]	Time [min]
-	60	2
20	100	2
4	132	0

The analysis duration was 14 min and the time required by the GC to cool down to the initial temperature was approximately 2 min. As a result the sampling frequency was approximately $1/16 \text{ min}^{-1}$. The retention times of the reaction mixture components are presented in Table 3.04. The separation of acetone and 2-propanol peaks was not possible with the present temperature program.

Table 3.04 Retention time of the reaction components for the online HP GC.

Component	Retention time
2-propanol	3.29 min
Acetophenone	8.24 min
1-(R)-Phenylethanol	12.34 min
1-(S)-Phenylethanol	12.75 min

The analysis of the results as well as the operation of the 6-way valve was performed with the HP-Chemstation Rev. A.07.01 software.

3.4.3. Offline gas chromatography

The online determination of the enantiomeric excess during the reaction was not possible due to the low detector sensitivity of the HP chromatograph combined with the very high enantioselectivity achieved at almost all studied reaction conditions. Therefore the samples collected at the outlet of the reactor during the reaction progress were analyzed offline in a Hewlett Packard 5890A (Santa Clara, USA) gas chromatograph situated at the Chair of Biotechnology, RWTH, Aachen,

focusing on the reaction products, 1-(*S*)- and 1-(*R*)-phenylethanol. Prior to measurement the samples were extracted with equal amount of ethyl acetate and distributed into GC vials.

The gas chromatograph was equipped with an FID detector, maintained at 220°C and a split/split-less injector, maintained at 180°C. The carrier gas was helium with a head pressure of 150 kPa. The separation was realized through an FS-CYCLODEX beta-I/P (25 m x 0.25 mm x 0.44 µm) GC column (CS-Chromatographie Service) at the temperature program presented in Table 3.05.

Table 3.05 Gas chromatography program for the offline GC analysis.

Rate [°C/min]	T [°C]	Time [min]
-	60	2
5	100	2
4	132	0

Table 3.06 Retention time of 1-(*R*)- and 1-(*S*)-phenylethanol in the offline HP GC.

Component	Retention time
1-(<i>R</i>)-Phenylethanol	17.64 min
1-(<i>S</i>)-Phenylethanol	18.10 min

The analysis of the results was performed with the HP-Chemstation Rev. A.07.01 software.

3.4.4. Experimental procedure

The reaction conditions, namely temperature, thermodynamic activity of substrates and water as well as total flow rate, were chosen. The thermoconstant chambers temperature was set, the valves behind the saturation flasks were opened and the flow rates of the dried nitrogen streams were accordingly selected and set by means of the LabView Program, operating the reactor. Initially the flow was

directed through the reactor by-pass. The GC sampling was initiated and the equilibration process of the reaction mixture was monitored.

In the meantime an amount of deposited enzyme preparation was introduced in the tubular reactor and stabilized between two glass wool layers. The resulting packed-bed was offline percolated with dried nitrogen, in order to remove possible excess water amounts. Subsequently, the reactor was mounted in the reaction unit and the packed bed was tempered at the reaction temperature, for approximately 30 min, before the reaction was initiated. When the mixture equilibration process was finalized, the mixture was switched to pass through the reactor. The reaction progress was followed by means of the online GC.

For the enantioselectivity studies, the sample collected at the reactor outlet during each experiment was extracted and analyzed by means of the offline GC.

3.4.5. Equilibrium calculations in the gas/solid reactor

It is assumed that the carrier gas (nitrogen), after bubbling for some time in the substrate saturation flask, was equilibrated with the liquid phase. Under this assumption, the amount of the substrate that was carried out of the saturation flask by the carrier gas corresponds to the saturation partial pressure of the specific compound at a defined temperature. The calculation of the saturation pressure of the two substrate compounds (acetophenone and 2-propanol) and water, at this temperature, was performed through the Antoine equation (Eq. 2.01).

The Antoine equation was implemented twice, for both the saturation and the reaction temperature, T_s and T_r respectively. The Antoine coefficients of the two substrates, the two products and water are included in Appendix B.

The flow rate of nitrogen (V_i , [mL/min]) entering each saturation flask was recalculated to molar flow rate ($Q_i^{N_2}$), by applying the following expression of the ideal gas law:

$$Q_i^{N_2} = \frac{P_{abs} \cdot V_i}{R \cdot T_s} \quad \text{Eq. (3.04)}$$

The compound molar flow rate of the stream leaving each saturation flask (Q_i^{out}) could be calculated, by means of the proportionality of the partial pressure, of the compound (p_i^s) and nitrogen ($P_{abs} - p$):

$$Q_i^{out} = Q_i^{N_2} \cdot \frac{p_i^s}{P_{abs} - p_i^s} \quad \text{Eq. (3.05)}$$

An additional carrier gas stream was used as 'make up gas' and its molar flow rate ($Q_{mug}^{N_2}$) was calculated again through the ideal gas law:

$$Q_{mug}^{N_2} = \frac{P_{abs} \cdot V_{mug}}{R \cdot T_s} \quad \text{Eq. (3.06)}$$

The total molar flow rate of the reaction mixture formed in the mixing chamber, resulted from the addition of the molar flow rates of the separate streams:

$$Q_{tot} = \sum_{i=1}^{NoComp} Q_i^{N_2} + \sum_{i=1}^{NoComp} Q_i^{out} + Q_{mug} \quad \text{Eq. (3.07)}$$

The partial pressure of each compound in the reaction mixture (p_i) was calculated through:

$$p_i = \frac{Q_i^{out}}{Q_{tot}} \cdot P_{abs} \quad \text{Eq. (3.08)}$$

The compound thermodynamic activity (α_i) could be calculated by dividing the partial pressure of the compound over its saturation pressure, at the saturation temperature T_s .

$$\alpha_i = \frac{p_i}{p_i^s} \quad \text{Eq. (3.09)}$$

The gas mixture was then directed to the reaction unit, where the temperature could be decreased, aiming at increasing the thermodynamic activity of the compounds. Under the assumption that the total pressure remained constant, the partial pressure of each compound remained also constant, whereas the saturation pressure decreased due to the decrease of the temperature of the system.

The total volumetric flow of the gas mixture entering the reactor (at reaction temperature T_r) could be calculated:

$$V_{tot} = \frac{Q_{tot} \cdot R \cdot T_r}{P_{abs}} \quad \text{Eq. (3.10)}$$

The aforementioned equilibrium calculations were used for setting the conditions of each experiment in the gas/solid reactor. For the target thermodynamic activities of the reactants and water, at the desired reaction temperature and the desired total volumetric flow rate, the carrier gas flow rates were calculated and set.

3.4.6. Saturation process

A model aiming at describing the saturation process of the carrier gas (N₂) with the reacting compounds and water, was constructed according to [Mayer et al., 2001] using Model Builder 3.1.5 by gPROMS®, Process Systems Enterprise, London, UK.

The model, predicting the time needed for a carrier gas bubble to become fully saturated with the compound vapors, was based on a number of assumptions: radial symmetry of the diffusion process, evaporation rate at the bubble edge much higher than diffusion rate, vapor and carrier gas behaving as mixture of perfect gases, constant bubble size, negligible diffusion of the carrier gas into the liquid phase (low liquid solubility), constant absolute pressure inside the bubble, carrier gas temperature equal to the liquid temperature, constant liquid temperature, constant diffusivity of vapor into the carrier gas and sufficiently low carrier gas flow rate to prevent formation of aerosolized liquid phase droplets [Mayer et al., 2001].

The assumed constant, throughout the rising time, bubble radius r_0 [m] of the carrier gas exiting the vent opening was calculated through:

$$r_0 = \left(\frac{3 \cdot D_h \cdot \sigma}{4 \cdot g \cdot \rho} \right)^{1/3} \quad \text{Eq. (3.11)}$$

where:

D_h	vent opening diameter	[m]
σ	surface tension	[kg/s ²]
g	gravity constant	[m/s ²]
ρ	liquid compound density	[kg/m ³]

The mass balance over the bubble radius, giving the partial pressure of the compound along the bubble radius was given by:

$$\frac{\partial p_i(r)}{\partial t} = D_{i,N_2} \cdot \left(\frac{\partial^2 p_i(r)}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial p_i(r)}{\partial r} \right) \quad \text{Eq. (3.12)}$$

for $r \in (0, r_0)$

Boundary conditions:

For $r = r_0$: $p_i = p_i^s$

For $r = 0$: $\frac{\partial p_i}{\partial r} = 0$

Initial conditions:

For $t = 0$ and $r \in (0, r_0)$: $p_i = 0$

For $t = 0$ and $r = r_0$: $p_i = p_i^s$

where:

p_i	partial pressure of compound i in the N_2 bubble	[mbar]
r	radial coordinate	[m]
D_{i,N_2}	diffusivity of vapors in N_2	[m ² /s]
p_i^s	saturation partial pressure of compound i	[mbar]

The degree of saturation Π over the bubble radius was calculated through:

$$\Pi(r) = \frac{p_i(r)}{p_i^s} \quad \text{Eq. (3.13)}$$

The gProms simulation file is included in Appendix C.

The time needed for the total saturation of the carrier gas bubbles with the vapors of the liquid compounds was calculated and compared with the rising time of the bubble through the liquid. A sufficient rising time is essential for the complete saturation of the carrier gas with the compounds and, therefore, applicability of the equilibrium calculations presented in section 3.4.5.

3.4.7. Analysis of the results

The reaction progress was followed by means of the online GC and the partial pressure of each reaction compound was calculated through the respective calibration curve. The conversion ξ (%) in the reactor for the two parallel reactions at any time point was calculated according to Eq.3.14 and Eq.3.15.

$$\xi = \frac{P_{PhEtOH}}{P_{AcPh_0}} \cdot 100\% \quad \text{Eq. (3.14)}$$

$$\xi = \frac{P_{Ac}}{P_{2-prop_0}} \cdot 100\% \quad \text{Eq. (3.15)}$$

where:

p_{PhEtOH}	1-phenylethanol partial pressure	[bar]
p_{AcPh_0}	acetophenone inlet partial pressure	[bar]
p_{Ac}	acetone partial pressure	[bar]
p_{2-prop_0}	2-propanol inlet partial pressure	[bar]

The specific reaction rate for the two reactions v [$\mu\text{mol}/\text{min}/\text{IU}$] was calculated according to:

$$v = \frac{Q_{AcPh}}{100 \cdot E_0} \cdot \xi \quad \text{Eq. (3.16)}$$

$$v = \frac{Q_{2-prop}}{100 \cdot E_0} \cdot \xi \quad \text{Eq. (3.17)}$$

where:

Q_{AcPh}	acetophenone molar flow rate in the reactor	[$\mu\text{mol}/\text{min}$]
Q_{2-prop}	2-propanol molar flow rate in the reactor	[$\mu\text{mol}/\text{min}$]
E_0	activity of deposited enzyme packed in the reactor	[IU]

The amount of active deposited enzyme used for the determination of the specific reaction rate was determined through a residual activity assay before the experiment.

A typical reaction progress curve is presented for enzyme deposited with sucrose in Fig. 3.02.

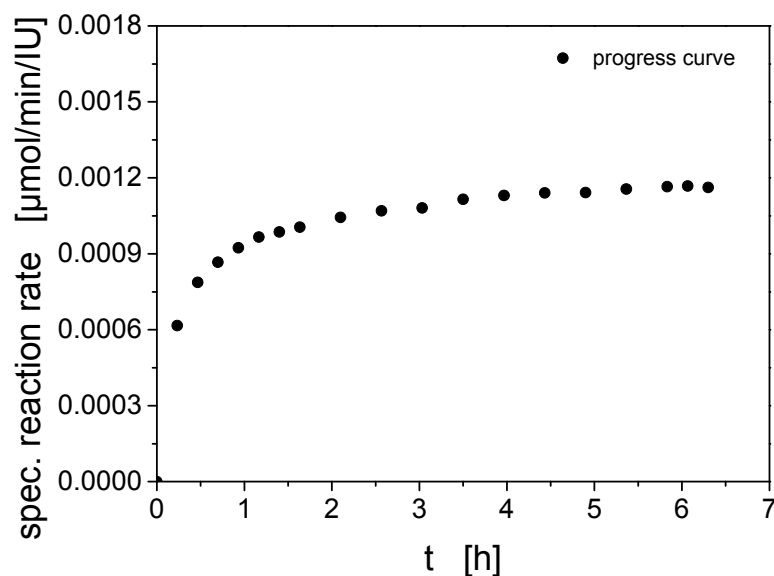


Figure 3.02 Typical progress curve of LBADH catalyzed conversion of acetophenone to 1-(R)-phenylethanol in a continuous reactor.

In the case of deposited enzyme preparations without sucrose, the deactivation of the enzyme could be already monitored within the first few hours of reaction, as it is indicated in Fig. 3.03.

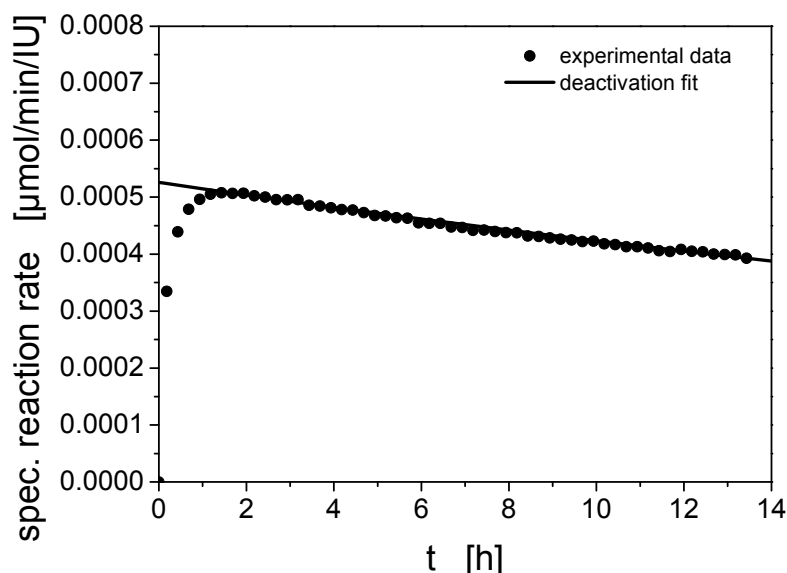


Figure 3.03 Typical progress curve with deactivation of LBADH catalyzing the conversion of acetophenone to 1-(*R*)-phenylethanol. The experimental data were fitted with a 1st order deactivation kinetic.

The progress curve was fitted with a 1st order exponential deactivation kinetic, given by Eq.2.06 and the specific initial reaction rate v_0 as well as deactivation constant k_d were determined.

The half life time $t_{1/2}$ of the enzyme in the gas/solid reactor was calculated through:

$$t_{1/2} = \frac{\ln 2}{k_d} \quad \text{Eq. (3.18)}$$

In the case of enzyme deactivation proceeding in two distinct phases, the time duration in which the enzyme activity was halved was designated as $t_{50\%}$.

For the enantioselectivity studies the enantiomeric excess (% ee) was calculated from the Eq.2.10. The concentration of (*R*)- and (*S*)-phenylethanol was calculated by the respective peak area by means of the calibration curve for each enantiomer.

3.4.8. Dispersion over the packed-bed

The dispersion of the reaction compounds along the packed-bed, in the absence of radial distribution and reaction, was modeled using Model Builder 3.1.5 by gPROMS®, PSE, London, UK. The model, predicting the response at the reactor outlet of a thermodynamic activity step or pulse at the reactor inlet, was developed under the assumption of uniform axial velocity and thermodynamic activity of the compound along the packed-bed diameter.

The linear velocity v of the gas mixture entering the reactor was calculated through:

$$v = \frac{\dot{V}_{tot}}{\pi \cdot r^2} \quad \text{Eq. (3.19)}$$

The saturation pressure p_i^s [mbar] of the reaction mixture compound i at temperature T [K] as well as its thermodynamic activity α_i was calculated through Eq.2.01 and Eq.2.02, respectively.

The mass balance of the reaction mixture compound i along the packed-bed, expressed in thermodynamic activities, corresponds to Eq.2.12 and is given by:

$$\frac{\partial \alpha_i(z)}{\partial t} = -\frac{\dot{V}_{tot}}{\pi \cdot r^2 \cdot (1-\varepsilon)} \cdot \frac{\partial \alpha_i(z)}{\partial z} + D \cdot \frac{\partial^2 \alpha_i(z)}{\partial z^2} \quad \text{Eq. (3.20)}$$

for $z \in (0, L)$

Boundary conditions:

For $z = 0$: $\alpha_i = \alpha_{i_0}$

For $z = L$: $\frac{\partial \alpha_i}{\partial z} = 0$

Initial conditions:

For $t = 0$ and $z \in (0, L)$: $\alpha_i = 0$

For $t = 0$ and $z = 0$: $\alpha_i = \alpha_{i_0}$

where:

r	packed-bed reactor radius	[m]
L	packed-bed reactor length	[m]
p_i	partial pressure of compound i in reaction mixture	[mbar]
ε	packed-bed voidage	[-]
D	dispersion coefficient of compound i	[m ² /s]

Mean residence time τ [s] of the reaction mixture compound i in the packed bed:

$$\tau = \frac{V_{packed-bed}}{\dot{V}_{tot}} \quad \text{Eq. (3.21)}$$

Reverse Peclet ($1/Pe$) number:

$$1/Pe = \frac{D}{L \cdot v} \quad \text{Eq. (3.22)}$$

where:

$\dot{V}_{tot}$	reaction mixture total volumetric flow rate	[m ³ /s]
$V_{packed-bed}$	packed-bed volume	[m ³]

The gPROMS simulation file is included in Appendix C.

3.5. Water and substrate adsorption

3.5.1. Water adsorption measurement unit

The measurement of the water adsorbed by the deposited enzyme preparation was performed in a water adsorption unit (Fig.3.04) which has an operation principle very similar to that of the gas/solid reactor [Lamare and Legoy, 1995]. Prior to this measurement, the enzyme preparation was dried by means of a pure N_2 stream until a totally dried enzyme preparation was obtained.

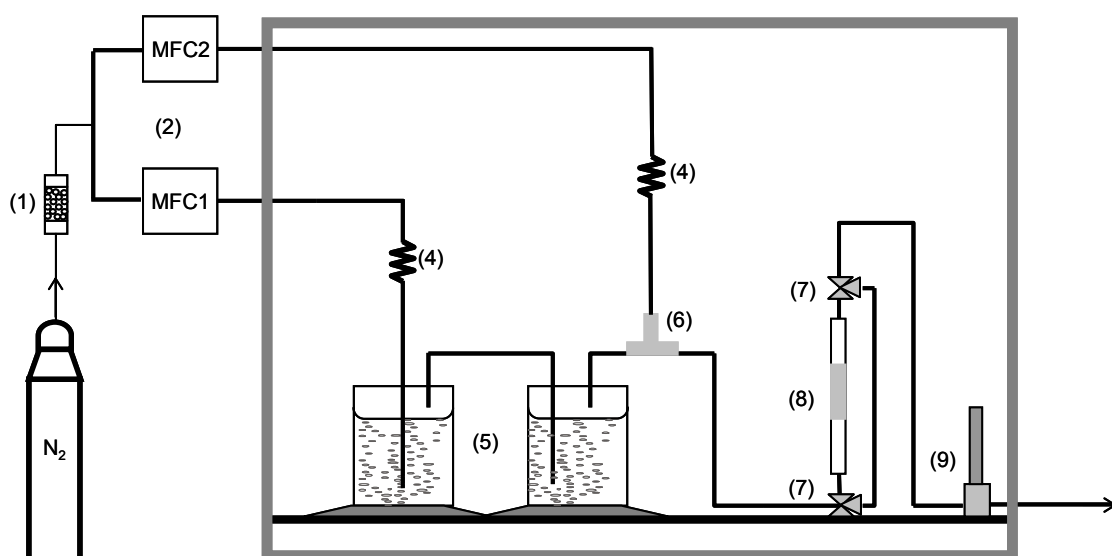


Figure 3.04 Water adsorption measurement unit: (1) humidity trap, (2) mass flow controllers, (3) thermo-constant chamber, (4) heating coils, (5) water saturation flasks, (6) mixing chamber, (7) 2-3-way valves, (8) packed bed, (9) humidity sensor

Two nitrogen streams, dried by means of a humidity trap (MT200-2, Agilent Technologies, Germany), were defined by mass flow controllers (F201C-FB-11-V, Bronkhorst Mättig GmbH, Germany) and directed to a thermo stated unit (WTB, Binder GmbH, Germany). One of the two streams was equilibrated with water in two successive water flasks. This stream was then mixed in a mixing chamber with the second stream of pure nitrogen to generate a mixture with total volumetric flow rate of 15 mL/min. This gas mixture was first directed

through the by-pass of the packed bed, until a constant humidity level was measured by a humidity sensor (D07P-EE22, E+E Elektronik GmbH, Bad Homburg, Germany). Then, the mixture was directed through the packed bed, containing a specific amount of immobilized enzyme preparation (19.8 mg and 120 mg lyophilized and immobilized enzyme, respectively, packed between two glass wool layers), by means of two 2-3-way valves (6604, Bürkert GmbH, Ingelfingen, Germany). The lowering of the water content in the mixture due to its adsorption by the packed bed was monitored by the humidity sensor. The adsorption isotherm was generated by repeating the aforementioned procedure in a stepwise manner for humidity levels from 10%RH to 90%RH. The desorption isotherm was acquired by the reverse procedure, i.e. by decreasing the humidity level stepwise from 90%RH to 10%RH. The operation of the water adsorption unit and data retrieval was achieved using a LabView (National Instruments, Texas, USA) program, created at the Chair of Biochemical Engineering, RWTH, Aachen.

3.5.2. Substrate adsorption measurement in the gas/solid reactor

The adsorption measurements of the two reaction substrates, acetophenone and 2-propanol, were conducted in the gas/solid reactor. During the adsorption experiments no reaction took place. This was achieved due to the absence of water in the adsorption measurements of individual substrates. In the case of competitive adsorption studies, with water present, the enzyme preparation was thermally treated before use (30 min at 105°C).

A gas mixture with a volumetric flow rate of 15 mL/min, unless otherwise stated, and a defined substrate thermodynamic activity was generated and equilibrated through the bypass. Subsequently, the mixture was directed through the reactor tube containing the deposited enzyme preparation (120 mg and 565 mg for acetophenone and 2-propanol adsorption studies, respectively, unless otherwise stated). The retention of the compound in the bed due to adsorption was monitored using an online GC. The procedure was repeated for stepwise increased substrate activities in the gas mixture.

3.5.3. Analysis of the results

A typical water break through curve is depicted in Fig.3.05. The nitrogen flow with 40% humidity, in this exemplary case, was directed through the packed-bed and the humidity of the stream, measured by means of the humidity sensor was reduced. The difference between the molar flow of water at the reactor outlet and the molar flow of water at the reactor inlet corresponded to the amount of water m_w adsorbed by the reactor and was calculated by Eq.3.23:

$$m_w = MW_w \cdot \int_0^t \frac{(\alpha_{w_in} - \alpha_{w_out}) \cdot p_w^s \cdot V_{tot}}{R \cdot T} \cdot dt \quad \text{Eq. (3.23)}$$

where:

MW_w	molecular weight of water	[g/mol]
V_{tot}	total flow rate	[mL/min]

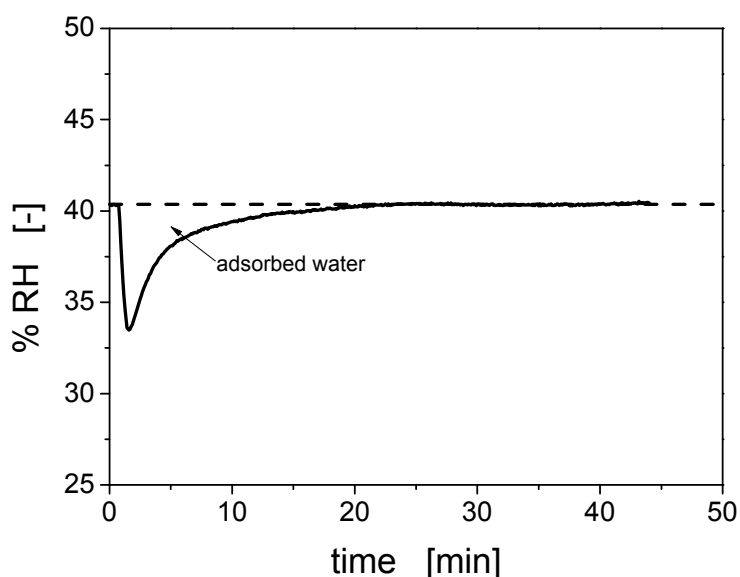


Figure 3.05 Water break-through curve.

The same methodology was followed for the determination of the substrates adsorption, using in this case the GC measurements.

The errors involved in the water adsorption measurement did not exceed 1% for the measurement of the lyophilized enzyme and 15% for the immobilized preparation. The substrates adsorption measurement involved negative errors of up to 10%.

The water monolayer was calculated with the linearized form of the Brunauer-Emmett-Teller (BET) [Brunauer et al., 1938] gas adsorption equation:

$$\frac{\alpha_w}{(1-\alpha_w) \cdot M} = \frac{1}{M_0 \cdot C} + \frac{\alpha_w \cdot (C-1)}{M_0 \cdot C} \quad \text{Eq. (3.24)}$$

where:

M	moisture content in w/w of dry solid	[%]
M_0	monolayer water coverage	[%]
C	constant	[-]

From the linear regression of the plot of $\alpha_w/((1-\alpha_w)M)$ versus α_w , the theoretical monolayer water coverage could be calculated:

$$M_0 = \frac{1}{(S + I)} \quad \text{Eq. (3.25)}$$

where S is the slope and I is the intercept [Costantino et al., 1997]

4. Results and Discussion

4.1. Reactor characterization

The constructed gas/solid reactor set-up had to be validated prior to its use for the reaction studies. Moreover, the reactor system itself, as well as the online GC had to be calibrated, to allow the quantification of the measurements. The full saturation of the carrier gas with the substrates and water in the saturation unit as well as the potential substrate or water condensation along the reactor set-up had to be investigated in order to figure out whether the thermodynamic control of the system was successful. The flow pattern along the reactor set-up as well as the mass transfer over the packed bed was described. In the following sections (4.1.1 to 4.1.6) these preliminary investigations, the description of the carrier gas saturation process, the flow pattern characterization and the description of the mass transfer along the bed are presented and discussed.

4.1.1. Reactor set-up operation and system validation

The gas/solid reactor could be operated within the range presented in the following table, depending on the capacity range of the used devices (mass flow controllers, thermoconstant chambers etc.). The operation range, for the experimental studies of the following sections, is also presented.

Table 4.01 Capacity and operation range of the gas/solid reactor.

Parameter	Capacity range	Operation range
Volumetric flow rate V [mL/min]	4-36	10-25
Temperature T [°C]	25-100	40
Relative humidity %RH [-]	0-90	40-70
Packed bed length L [cm]	0-20	0.3-3
Deposited enzyme amount m [g]	0-3.3	0.04-0.50

With respect to the total volumetric flow rate of the gas mixture in the reactor, care was taken that each one of the four mass flow controllers used was not operated below 10% or above 90% of its capacity. In this way an accurate flow control (within +/- 4-5% of the set value) was achieved for all operation conditions tested. The volumetric flow rate range of the studies was between 10 and 25 mL/min. Since most of the performed studies required absence of mass transfer limitation, this relatively low flow rate of the reaction mixture was compensated through the use of a low amount of deposited enzyme.

The thermoconstant chambers used could control the temperature down to 5°C above room temperature and up to 100°C with a minimal deviation of $\pm 0.5^\circ\text{C}$. Nevertheless, the operation temperature of the entire set up was restricted by the heating capacity of the thermoconstant water bath, used to temper the connection pipe between the two units. The studies presented in this work were all performed

at 40°C (reaction temperature), with a temperature difference of 5°C with respect to the saturation unit. The connection pipe was tempered at 45°C. The efficiency of its tempering at higher temperatures should be investigated.

Prerequisite for the successful thermodynamic control of the system was the accurate control of the flow rate of the carrier gas streams, the full saturation of the carrier gas with all compounds (the two substrates and water) and the effective temperature control over the entire reactor set-up, that would exclude condensation phenomena and would allow constant thermodynamic activities of the compounds. Due to the fact that the GC, that quantified the amounts of the reacting compounds, needed to be itself calibrated using the reactor, the applicability of the equilibrium calculations (section 3.4.5) and thus the informational on thermodynamic control of the system was investigated by an iterative method, described in the following paragraphs.

The accurate flow rate control was achieved through regular offline re-calibration of the mass flow controllers with air, as testing gas. With the calibration curves of the mass flow controllers in hand, the calibration of the GC for the reactants and products was performed, by creating gas mixtures of pre-calculated (Eq.3.04 - Eq.3.09) partial pressures of each compound with nitrogen in the saturation unit.

4.1.2. Saturation process

The saturation of the carrier gas bubbles in the saturation flasks of the two reactants (acetophenone and 2-propanol) and water is described in section below.

After the formation of the carrier gas bubble at the vent outlet, where the velocity of the bubble is considered equal to zero, the bubble started rising, with accelerating velocity, in the liquid. During the rising of the bubble, diffusion of the vapors of the liquid to the nitrogen took place. Spherical symmetry of the diffusion was assumed (Fig.4.01). Prerequisite for the complete equilibration of the carrier gas with the liquid is that the vapor pressure of the liquid in the carrier gas becomes equal to its partial saturation pressure at the temperature at which the

equilibration takes place. Therefore, the entire carrier gas amount contained in the bubble should acquire the partial saturation pressure of the liquid, even at its center. This was considered the indication of full saturation.

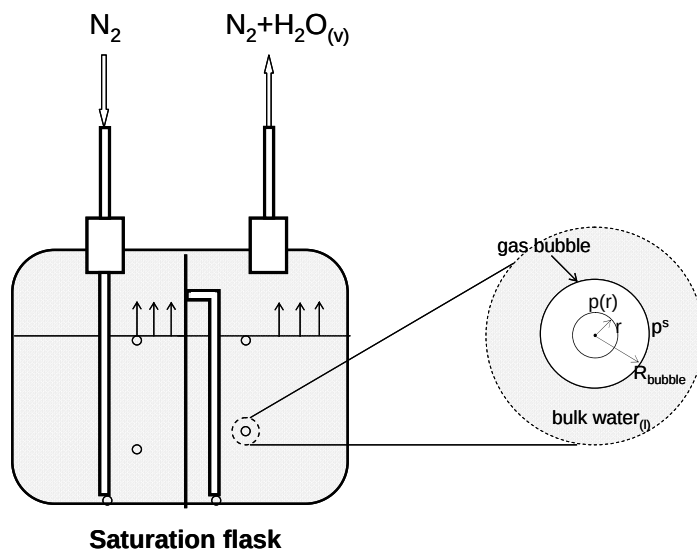


Figure 4.01 Schematic representation of the saturation process of nitrogen with water vapors in the saturation flask. Diffusion of water vapors from the bulk liquid to the nitrogen bubble with radial symmetry was assumed.

Initially the bubble size of carrier gas in each liquid was calculated, taking into account the physical properties of the respective compounds (Table 4.02) and the vent-opening diameter ($D_h=1.5\text{mm}$). Subsequently, the time required for the partial pressure of the compound to reach the saturation partial pressure (calculated according to the Antoine equation for the saturation temperature $T=45^\circ\text{C}$) at the center of the bubble, indicating in this way the time point of full saturation of the carrier gas with the respective compound, was calculated. The results, obtained by the model of section 3.4.6 based on the assumptions presented in the same section, are represented by means of the saturation degree along the bubble radius, for different durations.

Table 4.02 Physicochemical properties of water, acetophenone and 2-propanol, required for the calculation of the saturation process of nitrogen bubbles with the compounds vapors [Mayer et al., 2001; Lide, 2008].

Properties	Water	2-propanol	Acetophenone
D_{i,N_2} [m ² /s]	28.4E-6	8E-6	12E-6
ρ [kg/m ³]	990.2	786	900
σ [kg/s ²]	0.0687	0.01896	0.03615
$p_i^s(T = 45^\circ\text{C})$	0.0948	0.1803	0.0022

The degree of saturation over the radius of the formed bubble of nitrogen in the water flask is depicted in Fig.4.02. It is indicated that full saturation of the bubbles took place very fast, within only 0.28 s.

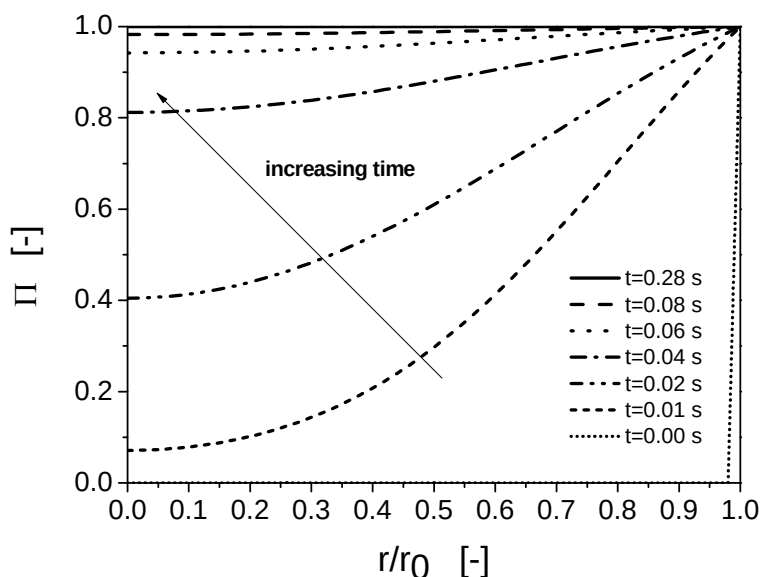


Figure 4.02 Degree of saturation of carrier gas with water vapors along the bubble radius, for different time points (0 s – 0.28 s). Simulation performed with gPROMS®, PSE, UK.

Since the rising time of the bubbles in the water flask was measured to be in the range of 1-2 s, even at higher volumetric flow rates and the saturation was additionally performed in the two compartment saturation flasks (Fig.4.02), where the contact time of the carrier gas with liquid was enhanced, full saturation of nitrogen with water was expected, according to the model, under all operation conditions tested. This result is in agreement with the equilibration experiments presented in section 4.1.3.

The degree of saturation of nitrogen with the two reactants, namely acetophenone and 2-propanol, along the bubble radius, for different time points, is depicted in Fig.4.03. Although the time till saturation was longer for 2-propanol and acetophenone, compared to water, it was also very short, in the range of 0.5 s and, therefore, complete saturation of nitrogen with these compounds was also achieved.

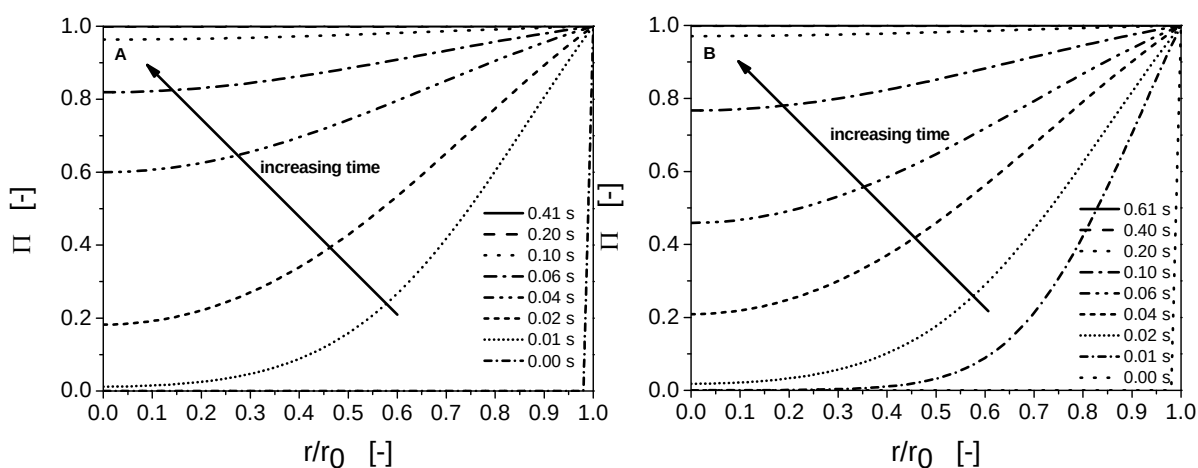


Figure 4.03 Degree of saturation of carrier gas with 2-propanol (A) and acetophenone (B) vapors along the bubble radius, for different time points. Simulation performed with gPROMS®, PSE, UK.

Finally, the radius of the nitrogen bubbles, formed in all three compounds, water, acetophenone and 2-propanol, as well as the time needed for the saturation of nitrogen in each of these liquids are summarized in Table 4.03.

Table 4.03 Radius of nitrogen bubble formed in water, 2-propanol and acetophenone, during the saturation process and duration until full saturation was achieved for each compound.

Parameters	Water	2-propanol	Acetophenone
r_0 [mm]	2	1.4	1.7
t [s]	0.28	0.41	0.61

4.1.3. GC calibration

For each reactant, several mixtures of increasing partial pressure of the compound with nitrogen were realized, by increasing the flow of nitrogen into the respective saturating flask and reducing the flow of the make-up gas, thus keeping the total flow constant. It was shown (Fig. 4.04) that by increasing the flow of nitrogen into the saturation flask, the achieved partial pressure was proportional to the flow entering the saturation flask and, therefore, full saturation was achieved even at higher flow rates. Under all experimental conditions tested, the expected partial pressure of both reacting compounds (acetophenone and 2-propanol) was achieved in the mixture which indicated a full saturation of carrier gas with these compounds and, therefore, the possibility to thermodynamically control the reaction mixture.

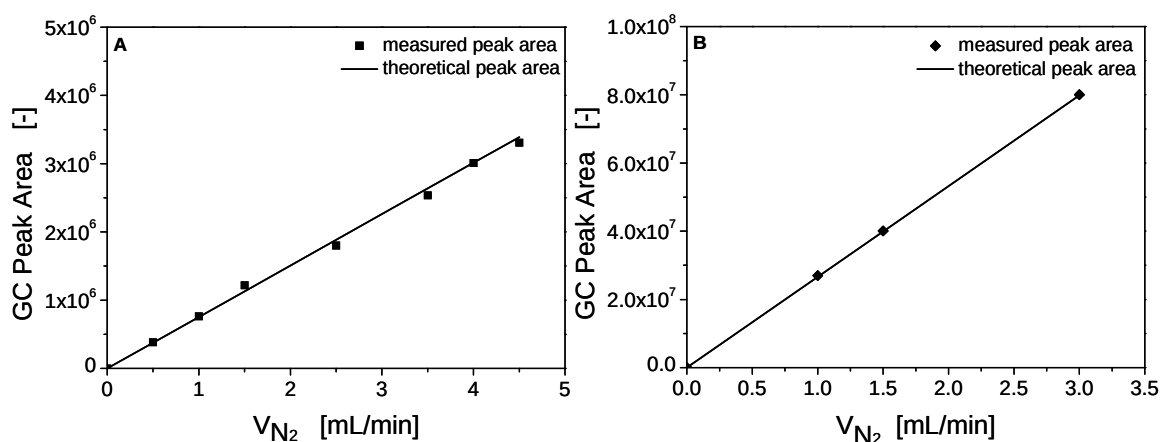


Figure 4.04 Theoretical and experimentally achieved GC peak area for acetophenone (A) and 2-propanol (B). The predicted values are reached even at higher flow rates of N_2 into the saturation flask, within the operational range.

For the calibration of the GC with the product 1-phenylethanol in particular, a different methodology was followed: the saturation of a nitrogen flow with this compound was directly performed in the reaction unit with a saturation flask integrated directly before the outlet of the reactor tubing to the GC. The saturated nitrogen with this compound at a constant temperature was directly measured in the GC which was calibrated in this way by means of the saturation partial pressure of 1-phenylethanol. The same process was followed also for acetophenone and it was verified that the calibrations performed in the two aforementioned ways gave the same results.

The equilibration of the mixture of nitrogen done in the saturation unit lasted approximately half a day, depending on the compound and its partial pressure). In contrast, the mixture equilibration in the reaction unit was very fast, lasting less than 2 hours (data in Appendix D). This different equilibration duration indicated pronounced adsorption of the reaction mixture compounds in the inner surface of the reactor pipeline, due to the extended heating coils used along the reactor set-up aiming at adequate heat transfer and temperature control.

4.1.4. Thermodynamic control

The parameter which expresses the compounds' availability to the enzyme and is thus decisive for the reaction rate is the thermodynamic activity. A constant reaction rate can only be achieved when both partial and saturation partial pressures are kept constant. The partial pressure of a compound in a realized mixture is retained constant when no condensation occurs, whereas the partial saturation pressure is retained constant when the temperature of the mixture is kept constant. Therefore, the constant reaction rates were an indication of successful control of the thermodynamic activity of the reacting compounds and, therefore, of a full saturation of the carrier gas, absence of condensation and last but not least successful control of the reaction temperature.

Whereas for the reaction compounds the validation of the respective partial pressures in the reaction mixtures could be achieved through the GC measurements, the water partial pressure and, therefore, the humidity of the reaction mixture was not measured. The commercially available humidity sensors are damaged in the presence of organic compounds and, therefore, the humidity in the mixture was set according to the calculations but not controlled during the experiments. Therefore, the reaction mixture relative humidity remained a “black box” for the system. Nevertheless, a similar investigation for the saturation of nitrogen with water was performed in the water adsorption unit, by means of the humidity sensor and full saturation at increased flow rates was confirmed (Appendix D). It was assumed that the results obtained in the water adsorption unit were applicable also to the reactor, due to the identical saturation principle in both set-ups.

The relative humidity of the gas mixture in the reactor set-up could be adjusted to up to 90% by a suitable combination of the mass flow controllers. Nevertheless, at high humidity values, above 70% and for reaction mixtures of a total volumetric reaction rate up to 20 mL/min, the reaction progress curves revealed unexpected condensation in the reactor, despite the fact that the partial pressure of water was far below its saturation partial pressure. The conversion was not smooth but fluctuated strongly, indicating formation of liquid water that led to high increase of the conversion and subsequent removal of this excess amount of water that led to a decreased conversion. Due to the lack of a humidity sensor, though, the investigation of this phenomenon was not possible and, therefore, no experiments were performed above 70% of relative humidity.

4.1.5. Flow pattern along the reactor set up

The flow pattern along the reactor set-up tubing as well as along the packed bed was characterized with the help of the non-dimensional Reynolds number (Re). The highest operational volumetric flow rate (25 mL/min) was used for this calculation. The physical properties of the gas mixture were approximated by the respective properties of nitrogen at 45°C [Perry and Green, 1997].

The calculation of Reynolds number was performed according to:

$$\text{Re} = \frac{\rho \cdot v \cdot d}{\mu} \quad \text{Eq. (4.01)}$$

where:

ρ	density of N ₂ at 45°C	[kg/m ³]
v	interstitial velocity of gas mixture	[m/min]
d	characteristic length	[m]
μ	dynamic viscosity of N ₂ at 45°C	[kg/m/min]

The interstitial velocity, through the reactor set-up tubing and packed bed was calculated by dividing the volumetric flow rate ($\dot{V}$) through the cross sectional area (A) of the tubing or the packed bed respectively. The characteristic length, in the case of the flow through the tubing is the inner diameter of the tube, while in the case of the packed bed is the particle diameter.

For the maximal volumetric flow rate used (25 mL/min), the value of Reynolds number for the flow through the tubing of the reactor set-up was calculated at 14, far below the critical value of 2000 for the onset of the turbulent flow. With respect to the flow through the packed bed, Reynolds number had a value of 0.4, much lower than the critical value of 10 which highlights the onset of turbulent flow through a packed bed [Ergun, 1952]. Therefore, the flow in the overall set-up was laminar, for the entire operational range.

4.1.6. Mass transfer over the packed-bed

The compounds' dispersion along the packed-bed reactor was characterized for typical operation conditions of the gas/solid reactor, by means of the dispersion model presented in section 3.4.8.

At the inlet of the packed-bed, of a typical length $L = 1$ cm and voidage $\varepsilon = 0.3$, a step of thermodynamic activity of acetophenone $\alpha_{AcPh_0} = 0.25$ was imposed at the time point 1 s in the gas mixture stream of total flow rate $V_{tot} = 14$ mL/min. The dispersion coefficient was assigned a value in the typical range of diffusion coefficients of gases, namely $D = 10^{-5}$ m²/s.

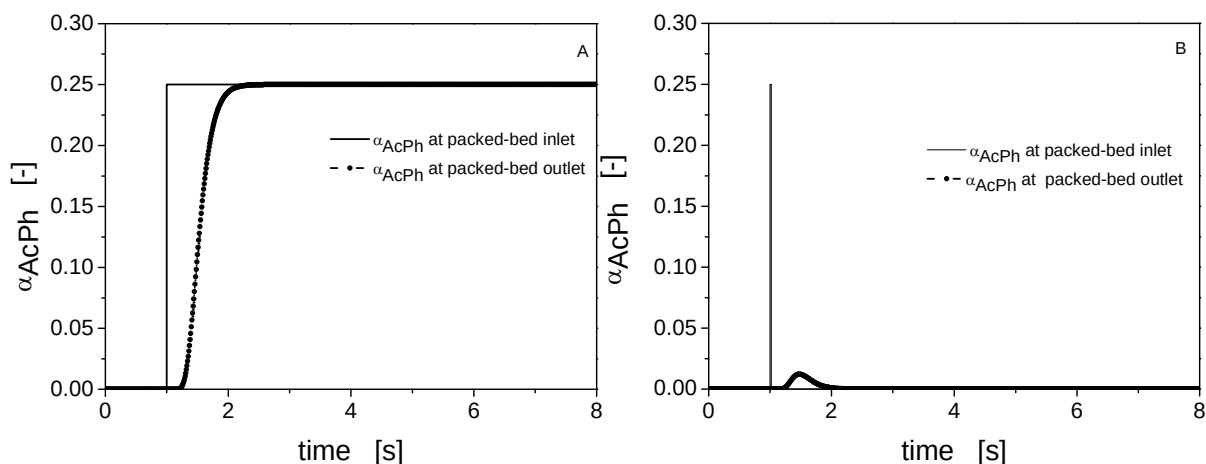


Figure 4.05 Simulation result: A) step response, B) pulse response. Simulation performed with gPROMS[®], PSE, UK.

The response at the outlet of the reactor (Fig.4.05A) indicated the presence of axial dispersion. If the reactor behaved as an ideal plug-flow reactor the expected response at the outlet would be a vertical line at time point $t = 1.84$ s. The time 0.84 s corresponds to the residence time in the packed-bed for the specific volumetric flow rate and reactor length. Nevertheless, the observed axial dispersion was limited. The reverse Peclet number (Eq.3.22) had a low value of 0.082. As a result, the initial activity of acetophenone was reached at the outlet within 2 s.

The presence of axial dispersion is also indicated in Fig.4.05B, by the result of the pulse response simulation. At the same conditions as those used for the step response simulation, the pulse of $\alpha_{AcPh_0} = 0.25$ imposed at the time point 1 s at the packed-bed inlet resulted in a broad residence time distribution at the packed-bed outlet. In the case of an ideal plug-flow reactor the response at the outlet would be a pulse at time point $t = 1.84$ s.

The time-space distribution, resulting from the step simulation is demonstrated at the following 3D diagrams generated by gPROMS.

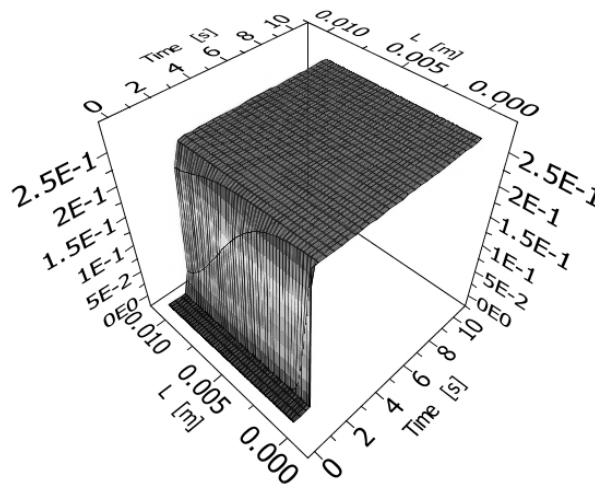


Figure 4.06 3D representation of simulation results: step response.

For increasing the packed-bed length or decreasing the total volumetric flow rate the dispersion would be increased. The initial thermodynamic activity imposed at the reactor inlet would be reached though within seconds, under all possible operation conditions. Thus, in all cases the calculated dispersion would not be experimentally followed due to the low sampling frequency of the online GC, which is approximately $1/15 \text{ min}^{-1}$.

4.2. Adsorption studies

The role of water in gas/solid reactions is pronounced. The thermodynamic water activity is the decisive parameter for both the activity and stability of the dried enzymes in these systems. Water is adsorbed to the dried enzyme preparation, hydrates it and renders it flexible and thus active. Like water, any other compound present will compete for adsorption sites on the biocatalyst [Yang and Russell, 1996a; Lamare et al., 2004].

While previous studies have focused exclusively on the adsorption of water equilibrating with the solid phase and the resulting effect on the enzyme activity and stability, there are no studies so far dedicated to the adsorption of substrates to the enzyme. There have been studies addressing the substrate adsorption [Hidaka and Matsumoto, 2000] or the possible product adsorption [Perez et al., 2007], without further quantification. In an effort to fill this gap, focus has been put upon the investigation of the adsorption of substrates on the biocatalyst, taking place in parallel or in competition to the water adsorption.

Initially, the water adsorption on the enzyme preparation was quantified indicating the effect of sucrose on the adsorption isotherm. Moreover, the adsorption of the two reaction substrates, namely acetophenone and 2-propanol, was investigated and quantified. The competitive adsorption, with respect to the water presence influence on the substrates adsorption, was also studied. The investigation of water and substrate adsorption was performed in a non-reacting system and, therefore, the phenomenon was individually studied.

4.2.1. Water adsorption to lyophilized enzyme

Initially, the water adsorption to lyophilized LBADH was studied and the water adsorption isotherm (Fig.4.07) was obtained. The water adsorption isotherm follows a pattern resembling the BET isotherm (Eq.3.24). Despite the fact that the assumptions of the BET theory (for non-polar adsorbate and inert surfaces) are not valid for the protein-water system studied, the theory is employed, though without conferring the physical meaning to the respective hydration parameters calculated.

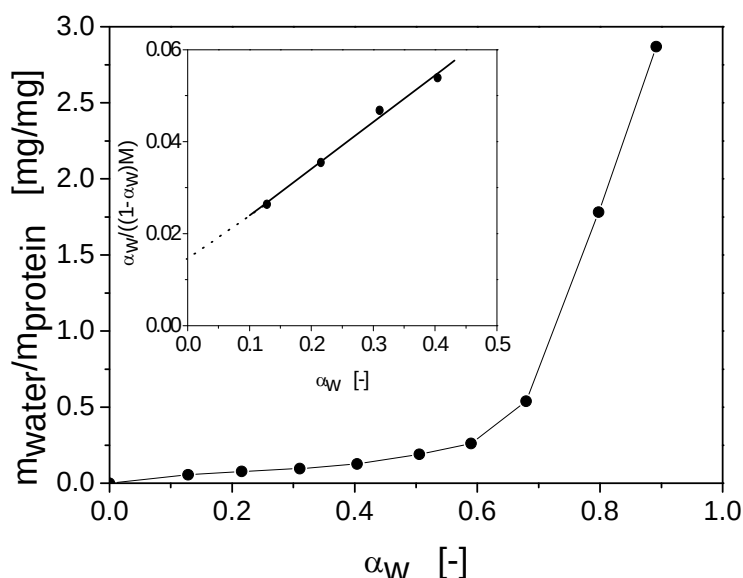


Figure 4.07 Water adsorption isotherm of lyophilized LBADH powder: $m_{\text{enzyme}}=19.8$ mg, $V_{\text{tot}}=15$ mL/min, $T=40^\circ\text{C}$. The insert shows the linearized BET isotherm according to Eq.(3.24).

Using the linear regression of the BET equation, the amount of water adsorbed, corresponding to the monolayer water coverage of the BET theory, was calculated to $0.0865 \text{ mg}_{\text{water}}(\text{mg}_{\text{dry protein}})^{-1}$. This value is in the same order of magnitude as the one reported for pharmaceutical proteins [Constantino et al., 1997] and various other proteins [Pauling, 1945].

The water adsorption isotherm of lyophilized LBADH differs significantly from the one of the yeast alcohol dehydrogenase (YADH) reported in previous studies: water adsorption to LBADH follows the pattern of the BET adsorption isotherm, whereas to YADH follows the Huttig isotherm pattern [Yang and Russell, 1996a]. LBADH also has much higher amounts of water adsorbed at water activity levels above 0.7, whereas the calculated amount corresponding to the monolayer water coverage is significantly lower than that of YADH ($0.35 \text{ mg}_{\text{water}}/\text{mg}_{\text{protein}}$). The water adsorption isotherm of the lyophilized enzyme powder depicts the capacity of the specific enzyme to adsorb water and depends on the amino acid composition of the protein and the polar side chains of the amino acid residues [Hnojewy and Reyerson, 1961; Brunauer et al., 1938] as well as the salts used during the lyophilization of the enzyme, in this case in an amount of 60% w/w. However, it provides no further information on the enzyme behavior in the gas/solid system. For this reason the adsorption behavior of the deposited enzyme preparation was further investigated.

4.2.2. Water adsorption to the deposited enzyme

The adsorption isotherm of deposited LBADH preparation was obtained in replicate (Fig.4.08). The amount of adsorbed water is referred to as the amount of water per protein amount, for facilitating the representation. Nevertheless, the amounts measured represent the cumulative adsorbed water by the immobilized preparation, including enzyme carriers, sucrose and salts used during the enzyme purification and immobilization. These components influence the water adsorption very strongly [Adlercreutz, 1991].

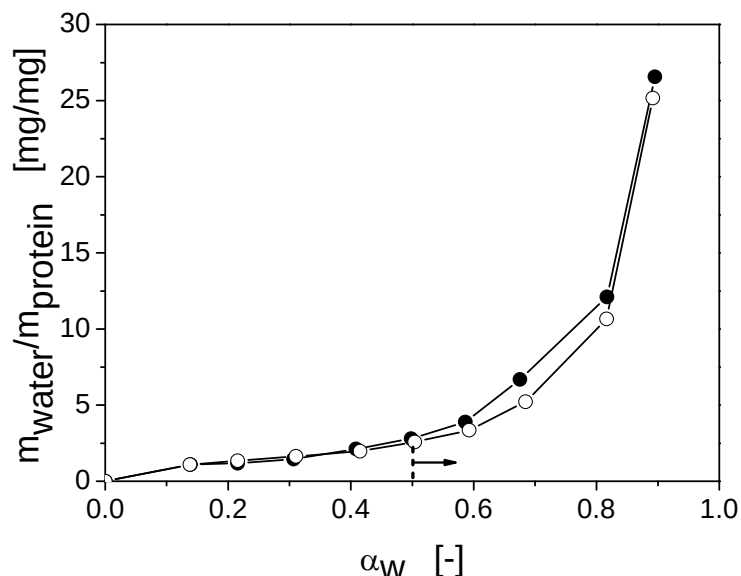


Figure 4.08 Water adsorption isotherm of immobilized enzyme preparation with sucrose. $m=120$ mg, protein loading=0.00275 mg/mg, $V_{\text{tot}}=15$ mL/min, $T=40^\circ\text{C}$. Open/close symbols refer to the two replicates. The arrow indicates the water activity level above which deviation from the initial adsorption behavior occurs.

The close proximity of the two isotherms indicates that the water adsorption measurement is reproducible, with a maximal error of 2%. Similar to the lyophilized LBADH, the adsorption isotherm also follows the pattern of the BET model. The water activity level above which water ceases being purely adsorbed by the immobilized preparation (point B, Fig.2.10) was identified to be slightly higher than 0.5. For the LBADH, it has been previously shown that it is preferable to operate the reaction system at water activities exceeding 0.5 in order to achieve increased conversion in the gas/solid reactor [Trivedi et al., 2005b]. This implies that higher conversion in the reaction system is actually achieved at a water activity level where only weaker adsorptive interactions with water occur.

4.2.3. Effect of sucrose on water adsorption

The beneficial effect of sucrose on the stability of the LBADH during the deposition process as well as on the storage and operational stability of the enzyme preparation has been shown previously [Ferloni et al., 2004; Trivedi et al., 2005a]. The effect of sucrose on the water adsorption to the deposited enzyme preparation has been, therefore, investigated here (Fig.4.09). For water activity levels exceeding the value of 0.5, up to which solely adsorption occurs (Fig.4.09A), the presence of sucrose in the enzyme preparation resulted in an increased amount of water adsorbed per unit of enzyme preparation. The hygroscopic nature of sucrose is responsible for the attraction of more water.

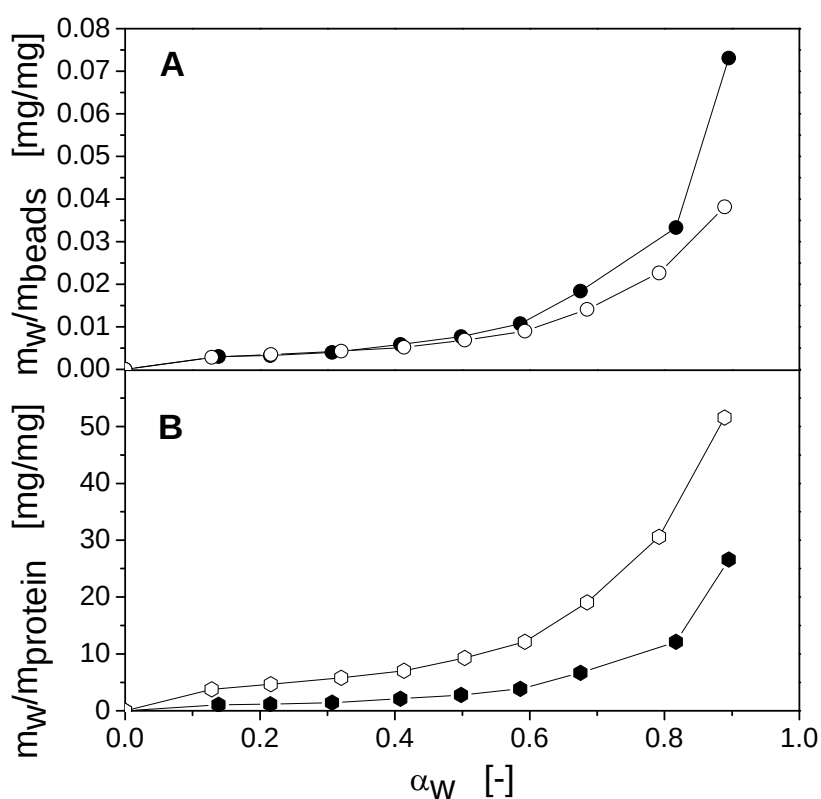


Figure 4.09 Sucrose effect on the water adsorption of deposited enzyme preparation. The water adsorbed is calculated here as water amount adsorbed per mg of beads of the catalytic bed (A) and as water amount adsorbed per mg of protein (B). The closed circles correspond to enzyme preparations with sucrose and the open ones without.

When comparing the two enzyme preparations, with and without sucrose, with respect to water adsorbed per unit of protein, the two curves were inverted (Fig.4.09B). This is due to the lower protein loading of the sucrose-free material, during the deposition process (protein loading of 0.00074 mg_{protein}/mg_{beads}), compared to the protein loading achieved in the case of sucrose containing material (protein loading of 0.00275 mg_{protein}/mg_{beads}). As a result, in the case of sucrose being present, more protein “sees” less water.

4.2.4. Hysteresis

The fact that the amount of water adsorbed by proteins when the equilibrium is approached from the “wet” side is higher than the amount of water adsorbed when the equilibrium is approached by the “dry” side has already been demonstrated [Mellon and Hoover, 1951]. This hysteresis has already been observed [Yang and Russell, 1996a; Killion et al., 1970; Mellon and Hoover, 1951; Bryan, 1987; Sirotkin and Faizullin, 2004] and was, therefore, compared in the current study between the lyophilized and the deposited enzyme preparation. The adsorption and desorption isotherms do not coincide for the lyophilized enzyme (Fig.4.10).

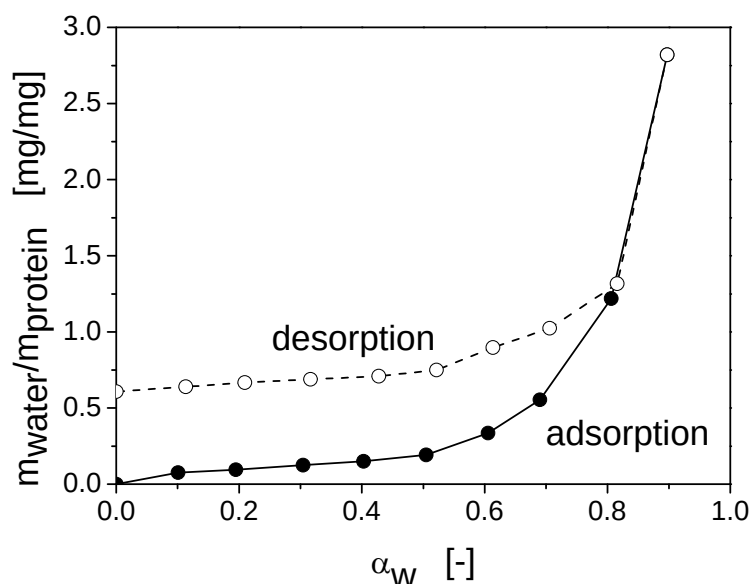


Figure 4.10 Water adsorption and desorption isotherms of lyophilized enzyme: $m_{\text{enzyme}}=19.8 \text{ mg}$, $V_{\text{tot}}=15 \text{ mL/min}$, $T=50^\circ\text{C}$.

The phenomenon of hysteresis is thought to occur due to alterations of the intra- and inter-molecular interactions of protein molecules during the hydration process [Hnojewy] and Reyerson, 1961; McMinn et al., 1993]. Hysteresis has also been attributed to the formation of hydrates during the protein hydration process. These hydrates persist at lower humidity levels during the desorption process [Mellon and Hoover, 1951]. In the case of LBADH it was found that water could not be desorbed entirely, even after drying the protein for many hours with anhydrous N_2 (data not shown). An unexpectedly high amount of irreversibly bound water of $0.6 \text{ mg}_{\text{water}}/\text{mg}_{\text{protein}}$ was measured. This may be attributed partly to the protein itself and partly to the TEA contained in the lyophilized enzyme (60% w/w). Observation of lyophilized protein particles that have adsorbed water revealed formation of agglomerates that appeared rigid and probably able to retain water. Regarding the deposited enzyme preparation, both with and without sucrose, the hysteresis was even more prominent (Fig.4.11).

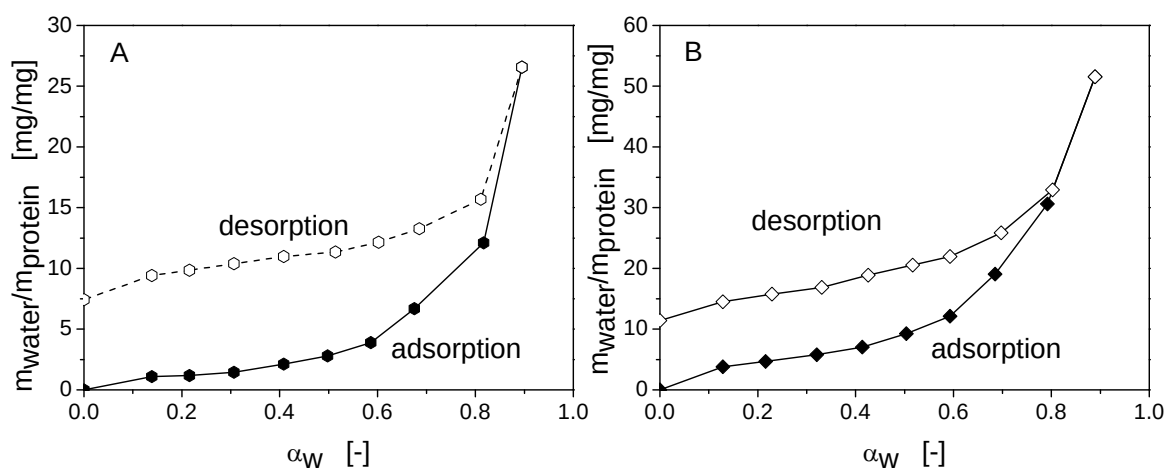


Figure 4.11 Adsorption and desorption isotherms of the deposited enzyme preparation with (A) and without (B) sucrose: $m=120 \text{ mg}$, protein loading= 0.00275 mg/mg (A), protein loading= 0.00075 mg/mg (B), $V_{\text{tot}}=15 \text{ mL/min}$, $T=40^\circ\text{C}$.

The methodology followed for the construction of the adsorption and desorption isotherms, involving the summation or subtraction of the adsorbed or desorbed respectively water amounts, on each activity level, caused error magnification. This was less pronounced for the adsorption isotherm, where the largest error at highest water activities amounted to less than 2%. However, in desorption, the errors increased predominantly at lower water activities and that led to an approximately 15% error in the determination of the irreversibly bound water. This

error, though large, does not change the overall shape of the adsorption and desorption isotherms.

In order to gain insight into the causes of hysteresis, SEM pictures of the enzyme preparations were taken. The glass carriers with the enzyme coating were observed before and after measuring the water adsorption (Fig.4.12). The enzyme distribution on the carriers' surface was dramatically modified during the process. The initially smooth layer of enzyme (Fig.4.12A and Fig.4.12C) was lost as the enzyme seemed to have been partly washed away from the carrier surface. Material bridges appeared between the individual carriers (Fig.4.12B) and clear areas on the carriers' surface were revealed (Fig.4.12D). This suggests that the biocatalyst is leached from the enzyme preparation to the glass wool layers.

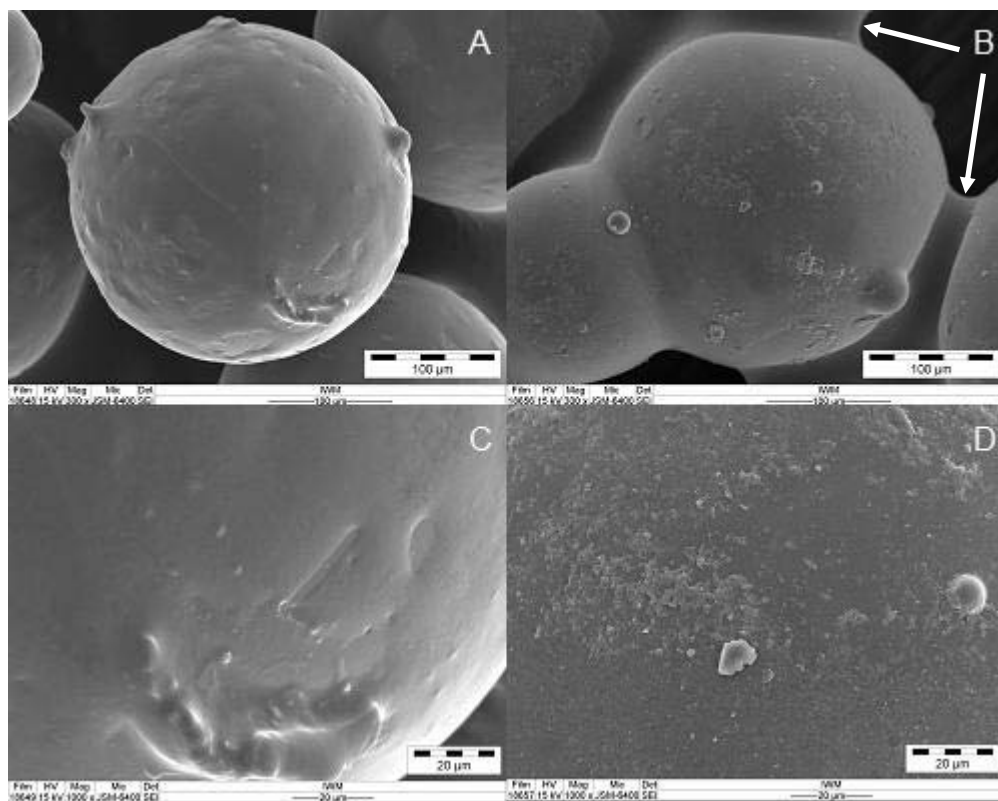


Figure 4.12 SEM pictures of the deposited enzyme with sucrose on glass carriers: (A) and (C) carriers before the water adsorption measurement process; (B) and (D) carriers after the water adsorption measurement process. The material bridges created between the individual carriers are indicated in (B) by arrows.

This is supported by the protein loading of the deposited preparation that was reduced to an approximately 30% lower value compared to that of the initial protein loading of the preparation. In the case of sucrose being absent during the immobilization the reduction of the protein loading was even more pronounced, reaching a level of around 45%. It appears that sucrose acts as a stabilizer for the attached enzyme on the carriers' surface.

4.2.5. Substrate adsorption

The dispersion model of acetophenone along the packed bed predicted that the dispersion of a gas mixture of acetophenone activity $\alpha_{AcPh} = 0.577$, with total volumetric flow rate $V_{tot} = 10$ mL/min and temperature $T = 40^\circ\text{C}$, for a packed bed of $L = 1$ cm length, and voidage $\varepsilon = 0.3$, assuming only axial dispersion with an axial dispersion coefficient of $D = 10^{-5}$ m²/s, would be limited. As it is depicted in Fig.4.13, the model predicted that the time needed under these conditions for the step of acetophenone activity, imposed at the inlet, to reach the packed bed outlet was approximately 3 s.

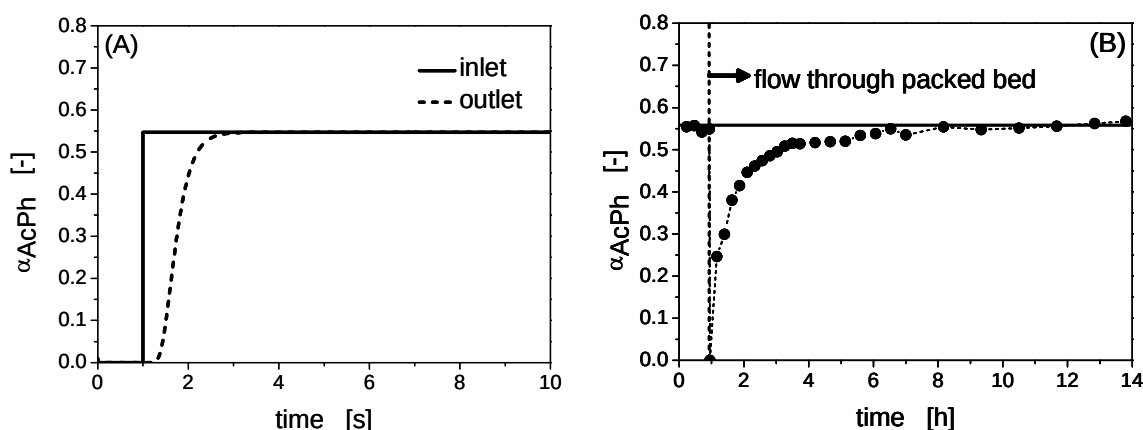


Figure 4.13 Response of an inlet acetophenone activity step at the packed bed outlet: $L=1$ cm, $\varepsilon=0.3$, $\alpha_{AcPh}=0.577$, $V_{tot}=10$ mL/min $T=40^\circ\text{C}$, $D=10^{-5}$ m²/s. (A) prediction through simulation performed with gPROMS®, PSE, UK, (B) experimentally

It would be impossible to monitor this limited dispersion, due to the low frequency of the GC sampling. Therefore if only axial dispersion took place along the packed

bed, experimentally we would expect to detect the final acetophenone activity immediately after the switching of the flow from the bypass to the reactor.

Nevertheless, by performing the respective experiment, choosing the same conditions assumed for the simulation, and switching the gas mixture from the bypass through the packed bed, it was observed that the outlet acetophenone activity was significantly reduced and restored back to its inlet value only after approximately 8 hours.

This observation led to the conclusion that acetophenone is retained through the packed bed due to adsorption. The adsorbed amount is significant and, therefore, the equilibration of the gas mixture may last for hours, which may possibly have a great influence on the reaction, at a reacting system, due to the concentration gradient created. Therefore, in order to fully understand the system it is essential to quantify the adsorption of not only water but also of the substrate.

4.2.6. Acetophenone adsorption to the packed bed with deposited enzyme

After turning the flow from the bypass to the packed bed, the partial pressure of acetophenone dropped as a result of its adsorption by the bed and a negative peak was observed over hours.

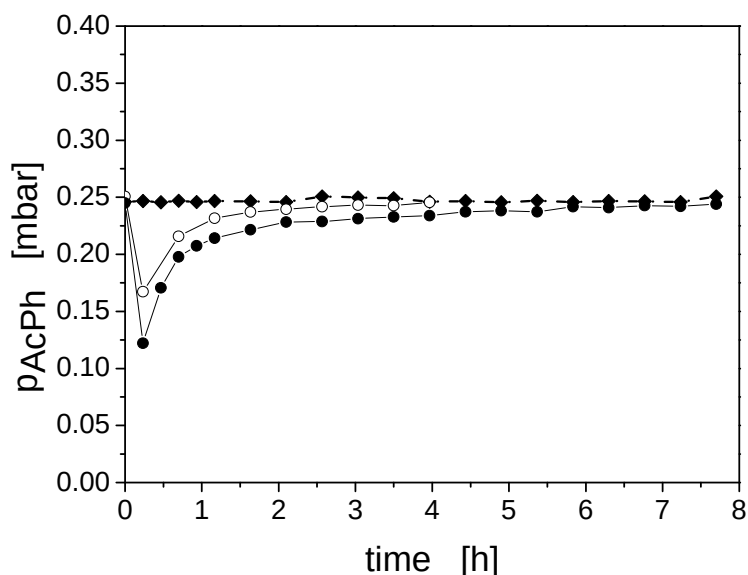


Figure 4.14 Effect of enzyme on the acetophenone adsorption capacity of the catalytic bed: $m=565$ mg, protein loading= 0.0021 mg/mg, $V_{tot}=10$ mL/min, $T=45^{\circ}\text{C}$. With diamonds the acetophenone partial pressure at the packed bed inlet, with open circles the partial pressure at the outlet of a packed bed with plain glass carriers and with closed circles the partial pressure at the outlet of a packed bed with glass carriers coated with deposited enzyme plus sucrose.

It was found that the amount of the adsorbed acetophenone to the carriers when enzyme was deposited on the surface was approximately 35% higher compared to the adsorbed amount to the plain glass carriers. The acetophenone adsorption isotherm was measured within the acetophenone activity range relevant to the operation of the gas/solid reactor ($\alpha_{AcPh}=0.05-0.32$) (Fig.4.15). Following a similar practice as in the case of water adsorption by the immobilized enzyme preparation, the amount of adsorbed acetophenone is referred to as amount of acetophenone per protein amount unit, for facilitating the representation.

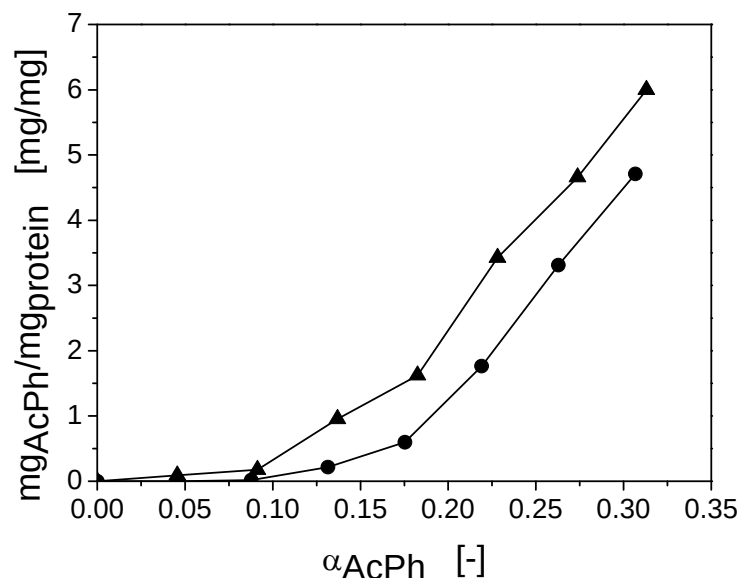


Figure 4.15 Acetophenone adsorption isotherms for the deposited enzyme preparation (with sucrose): with triangles the adsorption isotherm of the gas mixture without water vapors, with circles the adsorption isotherm with water vapors of $\alpha_w=0.54$. For both $m=120$ mg, protein loading=0.0021 mg/mg, $V_{\text{tot}}=15$ mL/min, $T=40^\circ\text{C}$.

Errors involved in the acetophenone adsorption measurements led mainly to underestimation of the adsorbed acetophenone amount due to limited tailing that summed up to 10%.

The amount of acetophenone adsorbed by the immobilized enzyme preparation is very low for acetophenone activities below 0.1. Above this activity level it shows an almost linear increase. By comparing the adsorption isotherms of acetophenone and water (Fig.4.08) for the same range of activities it is clear that: (a) the pattern of the two isotherms is different; unlike the water adsorption isotherm, the acetophenone adsorption isotherm does not follow the BET pattern (Figure 2.10) and (b) the mass of adsorbed acetophenone is higher than the mass of adsorbed water at the same levels of thermodynamic activity. At the highest acetophenone activity level tested, namely $\alpha_{\text{AcPh}}=0.32$, the adsorbed acetophenone amount reached a value of 6 mg_{AcPh}/mg_{protein}. This amount of water is adsorbed only at water activity levels above 0.6. In terms of mole amounts, the adsorbed amount of water is almost double the adsorbed amount of acetophenone (0.091 mmol_{water}/mg_{protein} versus 0.05 mmol_{AcPh}/mg_{protein}), for the same level of thermodynamic activity of the two compounds, namely 0.32.

4.2.7. 2-Propanol adsorption to the packed bed with deposited enzyme

The adsorption of the second substrate, 2-propanol, to the deposited enzyme preparation in the gas/solid reactor was also investigated. In the dynamic experiment, gas mixtures of nitrogen and 2-propanol, with thermodynamic activities in the range of $\alpha_{2-prop}=0.079-0.38$, were directed through the packed bed but no negative peaks were detected (Fig.4.16). The GC sampling of the mixture took place only 6 min after the flow was switched from the bypass to the reactor. Even if adsorption of this compound occurred, it was not detectable in the specific set-up.

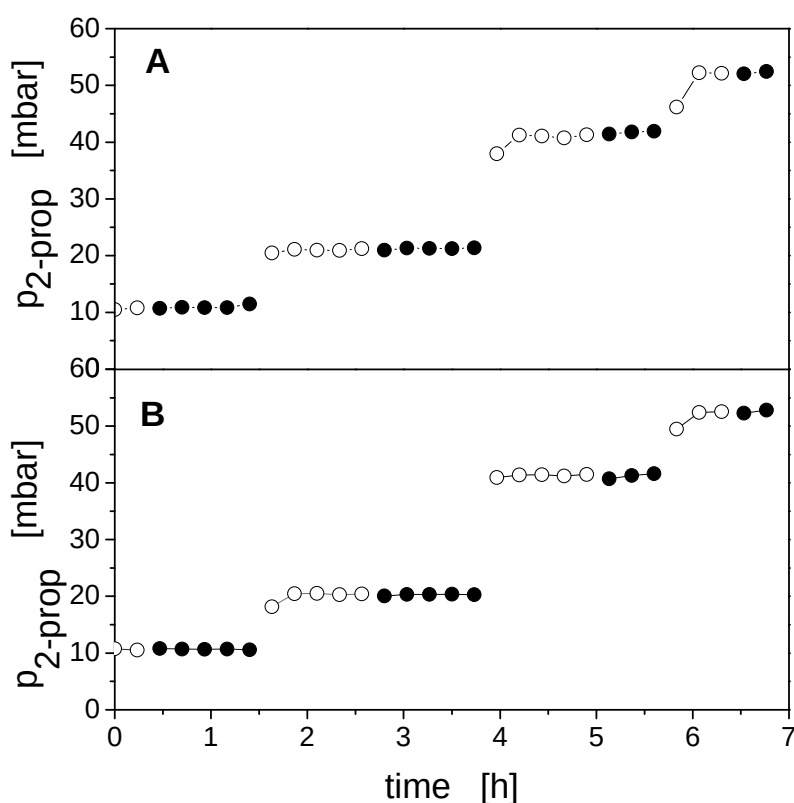


Figure 4.16 2-Propanol equilibration without (A) and with (B) water: $m=565$ mg, protein loading=0.0021, $V_{tot}=10$ mL/min, $T=40^{\circ}\text{C}$, $\alpha_w=0.54$. Open circles correspond to the flow through the bypass while closed ones flow through the packed bed.

4.2.8. Competitive adsorption of substrates and water

A potential effect of water on the adsorption of acetophenone and 2-propanol to the deposited enzyme preparation for a common water activity level for the reaction mixture in the gas/solid reactor of 0.54 was also investigated. All other parameters were kept at the same level as those in the investigation of the substrate adsorption without water in the mixture. The enzyme preparation was thermally treated, aiming at the cofactor deactivation assuming that this short thermal treatment did not severely alter the enzyme capacity to adsorb water.

By comparing the adsorption isotherms of acetophenone in Fig.4.15 it is obvious that the presence of water in the gas mixture leads to a decrease in the amount of acetophenone adsorbed by the enzyme. The substrate and water molecules compete for adsorption sites of the immobilized catalyst and that leads to a lower acetophenone adsorption. On the other hand, the presence of acetophenone molecules may also influence water adsorption. According to [Yang and Russell, 1996a] however, due to the low polarity of the acetophenone this effect is not expected. Due to the incompatibility of the humidity sensors with acetophenone, the respective competitive adsorption studies could not be performed.

Under the assumption that the water adsorption isotherm is not strongly influenced by the adsorption of acetophenone, the quantity of adsorbed water, at $\alpha_w=0.54$, was obtained, from the water adsorption isotherm (Fig.4.08). Similarly, the quantity of adsorbed acetophenone, at $\alpha_{AcPh}=0.22$, was also calculated. The ratio of adsorbed water moles to adsorbed acetophenone moles was found to be approximately 12.5 ($0.183 \text{ mmol}_{\text{water}}/\text{mg}_{\text{protein}}$ versus $0.015 \text{ mmol}_{\text{acph}}/\text{mg}_{\text{protein}}$), at the above mentioned water and acetophenone activity levels, which are typical values for the reactor operation. Taking into account the molecular weight of the LBADH (106 kDa), it is estimated that, at these conditions, 1 molecule of protein is surrounded by 19400 molecules water and 1560 molecules acetophenone.

As it is shown in Fig.4.16B, the presence of water did not influence the adsorption behavior of 2-propanol. Once again, 2-propanol adsorption by the immobilized enzyme preparation was not detectable in the dynamic experiment, exactly as in the case of water being absent. On the contrary, the presence of 2-propanol probably influenced the water adsorption. Previous studies [Yang and Russell, 1996a; Lamare et al., 1997; McMinn et al., 1993] have reported the suppression of enzyme hydration in the presence of small ketones and alcohols, such as acetone and n-propanol.

4.3. Enzyme hydration and activity

The lowest water activity level, at which detectable enzyme activity in the gas/solid reactor was observed, was determined. The influence of the water activity on conversion was investigated. The water adsorption at each level of water activity tested, depicted in the water adsorption isotherm was used as reference for correlating the reactivity of the enzyme with its hydration. For this study, deposited cell extract with sucrose was used. For each water activity tested, starting from 0.2 and increasing stepwise up to 0.65, a new sample of enzyme preparation was introduced in the reactor and the reaction was initiated.

From Fig.4.17 it becomes clear that for water activity levels below 0.4 the conversion was very limited, below 0.1%. Above this water activity level, which coincided with the activity level above which water ceased being purely adsorbed (adsorption isotherm deviating from the initial linear region), an exponential increase in the reaction conversion was observed.

The activity level at which enzymatic activity in the reactor started was hard to define, but was most probably between 0.2 and 0.25 of water activity, as it is indicated in the insert of Fig.4.17. The difficulty in this determination did not rise from the detection limit of the GC, which can detect conversion as low as 0.001% for this system, but rather from the remaining amounts of product in the reactor. Traces of phenylethanol remain adsorbed in the inner surface of the pipeline of the reactor even after thorough cleaning with nitrogen. As a result the produced phenylethanol at very low conversion could not be easily distinguished from these traces.

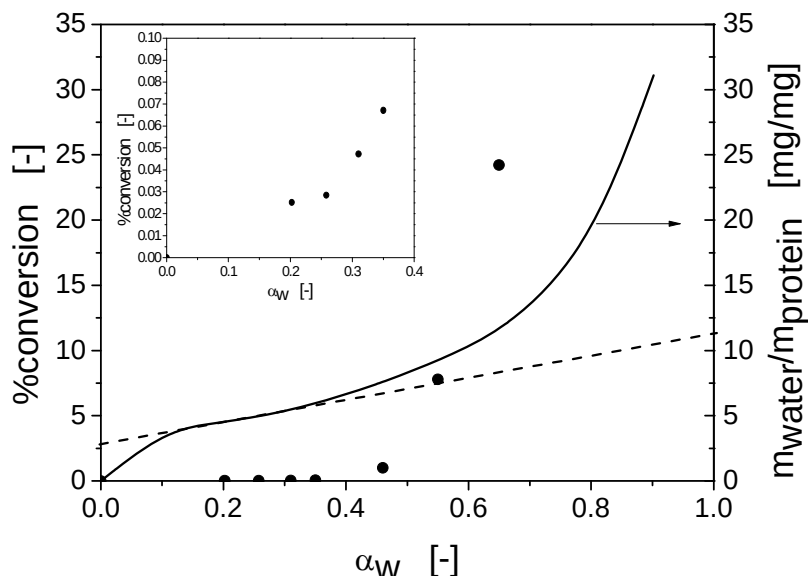


Figure 4.17 Water activity influence on the conversion and adsorption: $m=100$ mg, $E_0=220$ IU/g, $\alpha_{AcPh}=0.22$, $n_{2-prop}/n_{AcPh}=60$, $V_{tot}=15.5$ mL/min, $T=40^\circ\text{C}$. The conversion is represented by the closed symbols and the adsorption isotherm by a line (also by data points provided in Appendix D).

The implications related to the determination of the minimal water activity are numerous and, therefore, this result can not be considered as generally applicable, but rather case specific. It can not be accepted as the absolute minimal requirement for water by the LBADH to become active, but the minimal water activity required in the specific reaction system, under the present operation conditions.

First of all, the enzyme is deposited on the glass carriers and, therefore, as it has been already clarified in the section 4.2.2, the hydration of the enzyme at each water activity level is strongly influenced by the presence of carriers and additives. In a different system (e.g. lyophilized non-immobilized enzyme) the hydration at the same activity level would be different, leading to a different interaction of enzyme and water molecules and, therefore, different water activity requirement for the onset of enzyme activity. The enzyme purification state (cell extract or lyophilized enzyme) plays also an important role for the enzyme hydration and therefore the onset of the reaction. According to the water adsorption isotherm for the deposited cell extract, at water activity of 0.2 the water adsorbed reached an

amount of 5 $\text{mg}_{\text{water}}/\text{mg}_{\text{protein}}$, more than double compared to the amount adsorbed by the deposited lyophilized enzyme (2 $\text{mg}_{\text{water}}/\text{mg}_{\text{protein}}$) indicated in Fig.4.08.

Other influencing factors are the packed-bed length, influencing the mass transfer in the reactor as well as the temperature. The absolute enzyme activity in the reactor can additionally play a role, mainly influencing the ability to detect the reaction at lower water activity levels.

In conclusion, the minimal water activity level, defined here at approximately 0.2 to 0.25 is below the water activity of 0.3, documented previously [Trivedi et al., 2006] as the minimal water activity for enzymatic activity onset of the deposited lyophilized LBADH, measured at 60°C. This discrepancy may be attributed to the aforementioned varying parameters. Additionally, the amount of water adsorbed by the deposited enzyme at this level can not be considered as the threshold for enzymatic activity of the LBADH, as previously documented for different enzymes [Kurkal et al., 2005; Dunn and Daniel, 2004; Yang and Russell, 1996a], due to the influence of the enzyme support in the present case.

4.4. Kinetic studies

The investigation of the reaction kinetics was performed at a water activity level of 0.51. At this moderate water activity level, the reaction rates were expected to be elevated, due to increased water adsorption by the enzyme preparation and thus easily measurable, even at reduced substrate thermodynamic activity. On the other hand higher water activity would lead to an exponential increase of the reaction rate, which is not desirable for the kinetic studies. The influence of the water activity on the reaction kinetics was not investigated and thus the kinetic parameters were obtained at a single level of water activity. The preliminary studies on the reaction progress and the diffusion limitation due to enzyme layer thickness were performed at a slightly higher water activity of approximately 0.55.

4.4.1. Reaction progress curve

During preliminary kinetic studies, it was shown that the reaction rate of the two concomitant reactions was approximately equal, with the progress curve of the reaction of 2-propanol to acetone reproducibly preceding that of acetophenone to 1-(*R*)-phenylethanol (Fig.4.18) in all reactions performed.

The equal reaction rates can be explained by the coupling of the two reactions through the cofactor regeneration system. The time shift of the 2-propanol reaction may have been due to the faster equilibration of this compound in the system. The adsorption studies (section 4.2.6) revealed pronounced acetophenone adsorption on the other hand, which might have, therefore, influenced the course of this reaction.

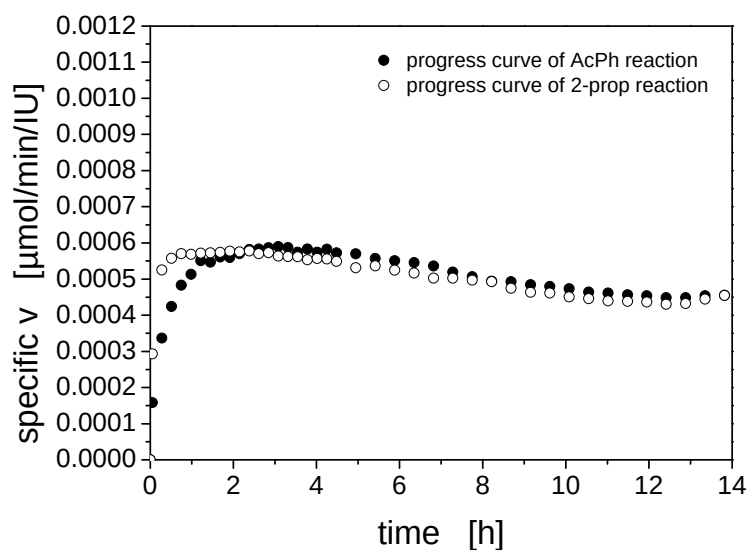


Figure 4.18 Progress curve of the two concomitant reactions: $m=80.7$ mg, $E_0=185$ IU/g, $\alpha_{AcPh}=0.155$, $n_{2-prop}/n_{AcPh}=83$, $\alpha_w=0.54$, $V_{tot}=10.5$ mL/min, $T=40^\circ\text{C}$.

The fluctuating reaction rate in both reactions might have been caused by the slow equilibration of the environment of the deposited enzyme, at the specific reaction conditions, of low volumetric flow rate ($V_{tot}=10.5$ mL/min). The hydration process of the deposited enzyme might have also played a role in the initially fluctuating reaction rate.

4.4.2. External mass transfer limitations

In order to obtain initial reaction rates, a kinetic study should be performed under differential operating conditions of the gas/solid reactor in the absence of external mass transfer limitation. To define the operational conditions under which no external mass transfer limitation would occur, the procedure as described below was followed. A constant amount of immobilized preparation was introduced in the reactor and the initial reaction rate was measured, for reaction mixtures of constant thermodynamic activity of reactants and water but varying flow rate. More specifically the flow rate was successively set to 29.8, 19.9 and 14.9 mL/min. In all cases the conversion remained below 1 %.

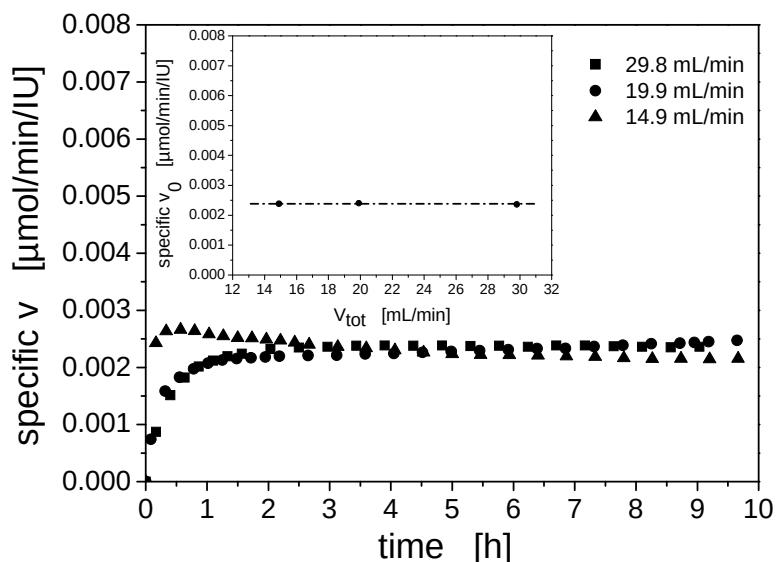


Figure 4.19 Progress curve at varied total flow rate with all other conditions kept constant: $m=40 \text{ mg}$, $E_0=46.5 \text{ IU/g}$, $\alpha_{\text{AcPh}}=0.21$, $a_{2\text{-prop}}=0.15$, $\alpha_w=0.566$, $T=40^\circ\text{C}$

The almost constant initial reaction rate achieved in all three cases (insert of Fig.4.19) revealed that in this range of flow rates the limiting step was the reaction and not the mass transfer of the compounds along the bed. If the system was mass transfer limited, the initial reaction rate would be reduced by reducing the flow rate.

4.4.3. Diffusion limitations

The standard immobilization process established by [Ferloni, 2004] involved the deposition of 170 IU on 500 mg carriers, producing deposited enzyme preparations of 340 IU/g specific activity. Nevertheless preliminary studies, performed with deposited enzyme preparations of lower specific activity (in IU/g_{carrier}), showed that the increase in the specific activity of the immobilization did not result in a proportional increase in the conversion achieved in the reactor, under the same operational conditions.

A potential effect of the thickness of the deposited enzyme layer on the resulting conversion in the reactor was studied, taking care that the study would be performed under reaction rate limiting conditions, to avoid potential external mass transfer limitation at higher deposited enzyme specific activities. Deposited enzyme preparations both with and without sucrose where tested.

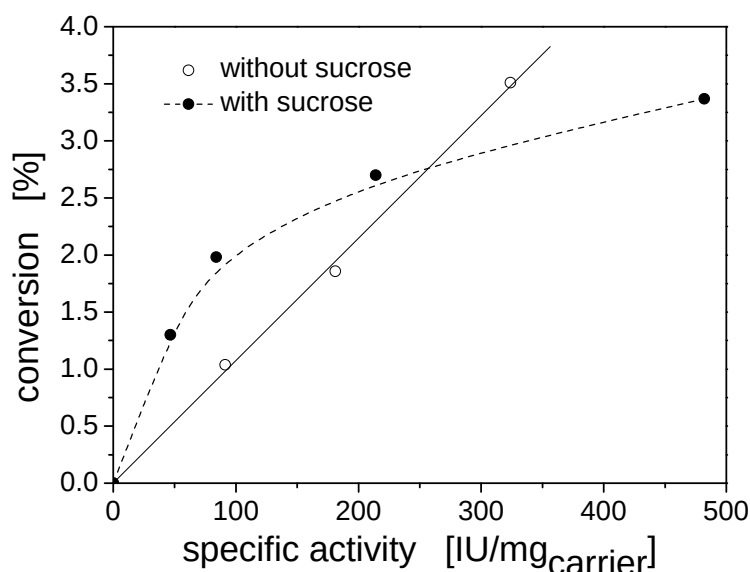


Figure 4.20 Conversion with deposited enzyme preparations of varying specific activity, with and without sucrose: $m=40$ mg, $\alpha_{AcPh}=0.21$, $\alpha_{2-prop}=0.15$, $\alpha_w=0.566$, $V_{tot}=29.8$ mL/min, $T=40^\circ\text{C}$

For this study, two initial enzyme solutions where created, with and without sucrose. They were subsequently diluted through 3 dilution steps, in 1:2 ratio each time. The resulting diluted solutions were deposited onto equal amounts of carriers.

The process led to deposited enzyme preparations with down to approximately one eighth of the initial specific activity, as verified through residual activity measurements. It was assumed that the specific activity in each case depicted the layer thickness of the enzyme deposited on the carriers. Higher specific activity would mean higher enzyme layer thickness on the carriers' surface.

In the case of deposited enzyme preparations without sucrose the increase in the specific activity resulted in a linear increase in the conversion reached in the reactor. On the contrary, in the case of the enzyme preparations with sucrose, by increasing the specific activity, lower than expected conversion was reached. Therefore, while at low specific activities, the conversion achieved by the deposited enzyme preparations with sucrose was higher compared to the conversion by the respective enzyme preparations without sucrose at higher specific activities the behavior was inversed (Fig.4.20). In all tested enzyme preparations the achieved conversion remained below 4 %.

This finding could indicate that in the case of the deposited enzyme preparation without sucrose the whole amount of enzyme was accessible by the substrates vapors and could be, therefore, used for the reaction, even at higher specific activity values. In the case of sucrose presence though, it seems that the dense layers of enzyme with sucrose rendered the deeper enzyme molecules inaccessible by the substrate molecules. Therefore, in this case not the whole amount of deposited enzyme was eventually used for the reaction, at increased specific activity values.

One other possible reason for the decreased conversion in the latter case might have been inadequate hydration of the deposited enzyme in the case of high specific activity deposited enzyme preparations with sucrose. The water adsorption studies of enzyme preparations with and without sucrose being present (section 4.2.3) revealed that the water amount adsorbed per protein amount was lower in the case of sucrose being present. The water adsorption studies, though, were performed at only one level of deposited enzyme specific activity and, therefore, the adsorption results in hand can not give an answer, with respect to the hydration at different specific activity levels.

4.4.4. Reaction kinetics

The investigation of the reaction kinetics in the present reaction system was complex. The deposited enzyme catalyzed two concomitant reactions, being coupled through the cofactor regeneration system. The cofactor was co-deposited with the enzyme on the carriers and like the enzyme itself, it was not free to move within the system. Therefore, a Uni-Uni mechanism could be assumed (section 2.3.1), whereas for initial reaction rates, product binding could be neglected and thus a standard Michaelis-Menten kinetic could be considered. A rather descriptive approach was favored for the kinetics description, because of the difficulty to quantify the enzyme accessible to the substrate molecules and the cofactor amount. An additional difficulty was the lack of knowledge, concerning the possible variable hydration of the enzyme at different levels of 2-propanol, as previously discussed (section 4.2.8).

Therefore, the thermodynamic activities of the two substrates, acetophenone and 2-propanol, were individually and simultaneously scanned in a range relevant for the reaction and the initial reaction rate achieved was measured and plotted against the respective substrate thermodynamic activity.

For the main kinetic studies, a low amount of 40 mg of deposited enzyme preparation with sucrose (42.5 IU/g_{carrier} deposited LBADH) was introduced each time in the reactor. The total flow passing through the reactor was set to 22.4 mL/min and the mean residence time was 0.127 sec. The reaction temperature was 40°C. The conversion remained below 1%, for all reaction mixtures tested.

Initially, the thermodynamic activity of both substrates was simultaneously varied by keeping their molar ratio constant at a value of 60 (Fig.4.21A). The acetophenone activity range scanned was from 0.12 to 0.28. The operation of the reactor at acetophenone thermodynamic activities lower than 0.12 or higher than 0.28, at the selected total volumetric flow rate (22.4 mL/min), would require the exchange of the mass flow controllers used and, therefore, was not performed.

Moreover, at operation at acetophenone activity values higher than 0.3, unexpected condensation phenomena were often observed as it has been also previously reported [Ferloni, 2004], that distorted the initial reaction rate measurement.

The initial reaction rate was found to be a linear function of the acetophenone activity within the studied range and could be described by a 1st order kinetic with respect to the acetophenone thermodynamic activity. The range of acetophenone activity studied was obviously very low and far below the saturating conditions for the enzyme. Therefore the maximal reaction rate and the Michaelis-Menten constant could not be individually determined for the gas/solid system.

The 1st order kinetic was fitted to the experimental data and the ratio v_{max}/K_m was calculated by the slope of the fitted curve, with the Michaelis-Menten constant having thermodynamic activity units.

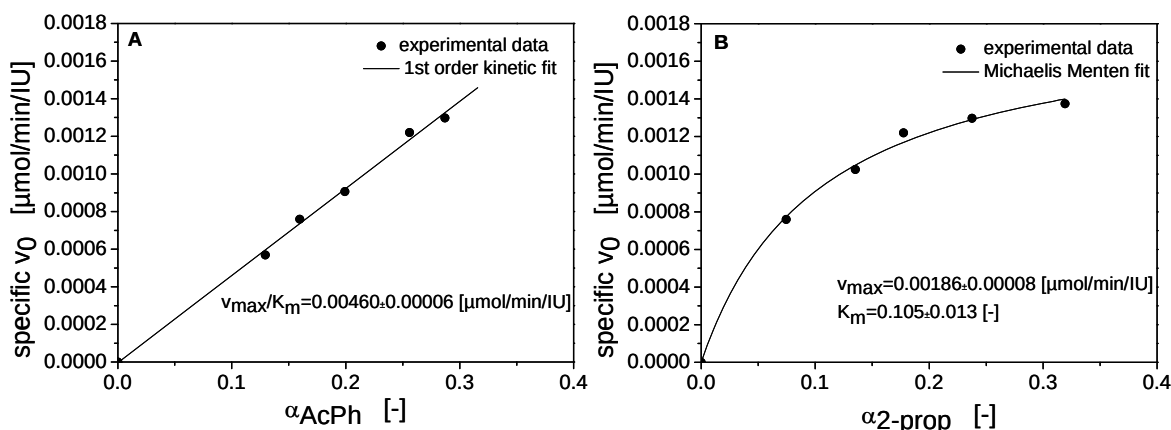


Figure 4.21 Dependency of the initial reaction rate on acetophenone and 2-propanol thermodynamic activity: A) variation of both thermodynamic activities, keeping a constant molar ratio: $\alpha_w = 0.51$, $n_{2\text{-prop}}/n_{\text{AcPh}} = 60$, $m = 40$ mg, $E_0 = 46.5$ IU/g, $V_{tot} = 22.4$ mL/min, $T = 40^\circ\text{C}$ and B) variation of 2-propanol thermodynamic activity: $\alpha_w = 0.51$, $\alpha_{\text{AcPh}} = 0.256$, $m = 40$ mg, $E_0 = 46.5$ IU/g, $V_{tot} = 22.4$ mL/min, $T = 40^\circ\text{C}$.

By varying the 2-propanol thermodynamic activity, while keeping the acetophenone activity constant at a value of 0.256, on the contrary, a Michaelis-Menten pattern was revealed. The increasing 2-propanol activity, corresponding to an increasing 2-propanol to acetophenone molar ratio (with the molar ratio taking

the values: 25, 45, 60, 80, 107), led to increasing initial reaction rates, approaching saturation.

A Michaelis-Menten kinetic was fitted and the maximal reaction rate (v_{max}) as well as the Michaelis-Menten constant (K_m) for 2-propanol was estimated. The calculated K_m value in thermodynamic activity terms ($K_m=0.1045$) would correspond to 0.55 mM recalculated in concentration terms, under the assumption of applicability of the ideal gas law.

The present results differ from the kinetic study results presented by Ferloni (2004) for the same reaction system. According to the previous study, the dependence of the initial reaction rate of acetophenone, on acetophenone activity, followed a Michaelis-Menten pattern. On the contrary, the thermodynamic activity of 2-propanol did not influence the initial reaction rate, in the molar ratio range of 40 to 110 of 2-propanol to acetophenone, for acetophenone activity constant at 0.05, and, therefore, enzyme saturation was assumed.

This discrepancy may be attributed to several factors. The first one is the enzyme source used. Within the present study, lyophilized purified enzyme was employed, while the previous study was performed with lyophilized cell extract. The presence of the additional constituents of the cell extract may not likely have influenced the intrinsic kinetic parameters of the reaction but might have influenced the water and substrates adsorption and also the hydration of the immobilized preparation. The water activity of the reaction mixture in the present study was also different (0.51 vs. 0.65) and since the role of water on the kinetic parameters has not been investigated for this system yet, these differences may be responsible for the varying kinetic studies results.

Additionally, the present study has been performed at kinetically controlled conditions, keeping the conversion very low, below 1%, for all acetophenone activities tested. On the contrary the previously reported kinetic studies were performed at conversion levels of up to 30%, while in the case of low acetophenone activities, conversion reached even 60%. The maximum conversion, allowed for kinetic studies, in previously reported studies was set to various levels,

ranging from 10% up to 30%. In order to study the intrinsic kinetics, the conversion is generally kept below 10% [Perez et al., 2007; Létisse et al., 2003; Lamare et al., 1997].

Finally, the influence of 2-propanol was measured at the present study at an elevated fixed acetophenone activity of 0.265, while the previously reported results were acquired for a very low acetophenone activity of 0.05 and high resulting conversion.

In general, opposing opinions can be found in the literature about kinetics in non-aqueous media. Most kinetic studies have shown that the enzyme behavior in non-aqueous media follows conventional models. However, the values of kinetic parameters may be very different from those of the same enzyme and reaction in aqueous media, with a general tendency for K_M to decrease with decreasing water activity [Bell et al., 1995].

For the gas/solid systems, on the contrary, it has been suggested that classical enzymology (i.e. Michaelian enzymology) can not be always used for the analysis of the gas/solid reactions [Lamare et al., 1997]. Nevertheless, Barzana and coworkers [Barzana et al., 1989] reported that the ethanol gas/solid transformation from alcohol oxidase followed the Michaelis-Menten kinetic with a K_M value almost two orders of magnitude lower than that found in aqueous solution. For the presently studied reaction system a comparison between the kinetic parameters in the gas/solid system and the respective aqueous system is not possible, due to lack of data, in aqueous media for the presently studied reaction system with co-factor regeneration.

More recent studies have elucidated the reaction mechanism of various gas/solid reaction systems [Bousquet-Dubouch et al., 2001; Perez et al., 2007], they have identified and quantified the inhibitory role of organic components [Létisse et al., 2003; Graber et al., 2007] or investigated the influence of water on the kinetic parameters [Graber et al., 2003]. Thus, they have demonstrated the possibility to derive the intrinsic kinetic parameters by means of the gas/solid reaction systems [Graber et al., 2008].

4.5. Stability studies

The operational stability of the deposited enzyme preparation in the gas/solid reactor was investigated. The studied influencing parameters were divided into catalyst preparation related and operating conditions related. The main part of the stability studies was performed with deposited enzyme preparations without sucrose. The enzyme stability data were partly correlated to activity data and the reproducibility of the measurements was proven.

First of all, the handling of the deposited enzyme preparation used in the gas/solid reactor was investigated as a possible factor influencing the stability in the reactor as well as the reproducibility of the measurements. The previously studied [Allison et al., 1999; Miroliaei and Nemat-Gorgani, 2001; DePaz et al., 2002; Trivedi et al., 2005] stabilizing effect of sucrose use as an immobilization additive was here proven. Moreover, the role of the state of the enzyme, related to the processing prior to its immobilization was elucidated. For this purpose the stability of deposited purified and lyophilized wild type LBADH was compared to that of deposited cell extract of wild type LBADH.

The influence of the reaction conditions on the operational stability of the deposited enzyme, namely the water activity of the reaction mixture, the acetophenone activity and the amount of deposited enzyme preparation, were investigated. The aforementioned investigation was performed with both the wild type LBADH and the variant G37D LBADH, allowing in this way a comparison between the operational stability of the two enzymes in the gas/solid reactor. The G37D variant, which prefers NADH, as cofactor, was tested in the gas/solid reactor as a promising candidate, due to the higher stability and lower cost of NADH compared to NADPH, as well as the higher thermostability of this enzyme compared to the wild type, when measured in solution.

4.5.1. Catalyst related parameters

During the stability studies the handling of the enzyme as well as the storage prior to its use in the gas/solid reactor emerged as two relevant parameters influencing the operational stability. While testing the reproducibility of the measurements, it became clear that the freshly immobilized enzyme samples gave different results, both with respect to activity in the reactor and stability, compared to the samples that had been stored for some time at 4°C and had been opened after the deposition process several times. The reduced residual activity due to enzyme aging and deactivation during storage was always taken into account by measuring each time the residual activity prior to use in the reactor and using always the expression of specific reaction rate (in $\mu\text{mol}/\text{min}/\text{IU}$) for the interpretation of the results. Nevertheless, this was not sufficient, since a totally different behavior in the reactor was revealed by fresh and old samples.

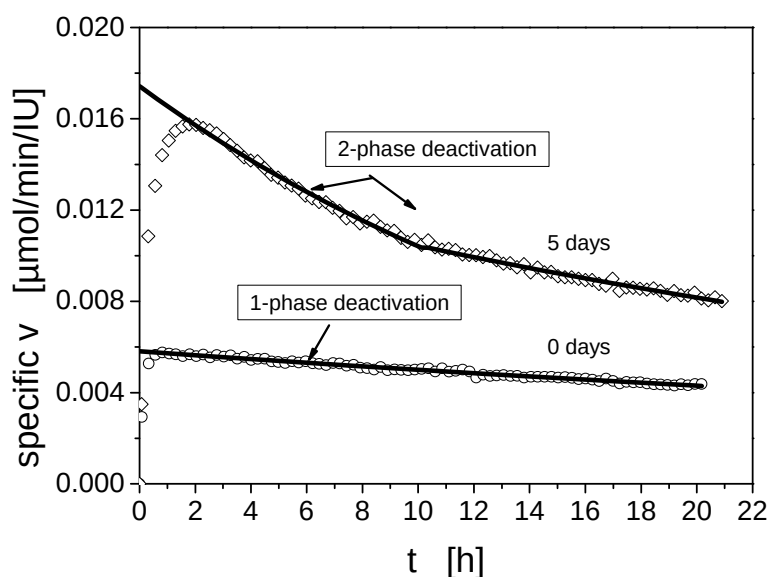


Figure 4.22 Operational stability of samples of deposited purified solution of G37D LBADH, originating from the same immobilization, tested after different storage durations: $\alpha_{AcPh}=0.21$, $\alpha_w=0.50$, $n_{2-prop}/n_{AcPh}=60$, $m=50\text{ mg}$, $V_{tot}=20\text{ mL}/\text{min}$, $T=40^\circ\text{C}$.

More specifically, the freshly immobilized enzyme samples used in the reactor revealed a relatively low initial specific reaction rate and one-phase deactivation, as it is indicated in Fig.4.22. On the contrary, when using another sample from the same enzyme preparation for a new measurement 5 days later, the initial specific

reaction rate reached was much higher and the deactivation in the reactor proceeded in two phases, with the first one being very fast, as it is indicated in the same figure, by the higher slope of the first phase of deactivation.

When testing several times in the reactor samples originating from the same immobilization, under the same reaction conditions, the behavior depicted in Fig.4.23 was revealed. The initial specific reaction rate was increased during storage and after some time decreased again. On the contrary the time until the reaction rate reached the half of its initial value ($t_{50\%}$) decreased with storage and after some time increased again (insert of Fig.4.23).

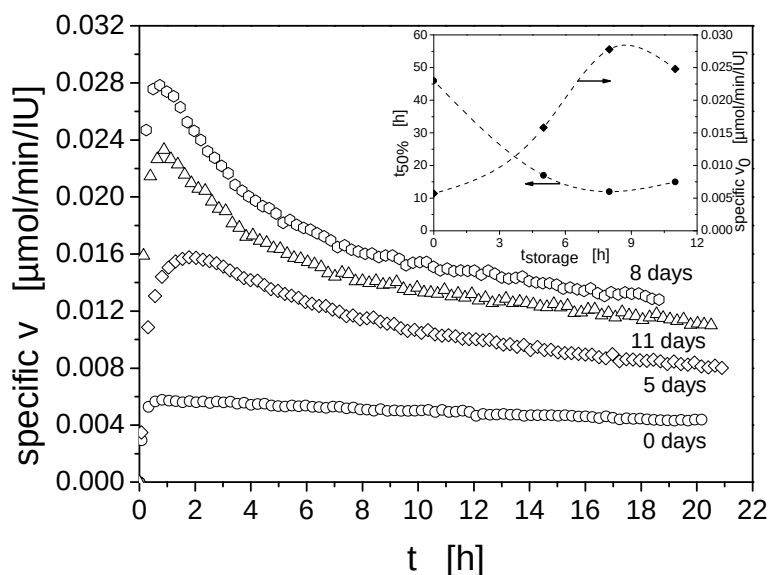


Figure 4.23 Operational stability of samples originating from the same immobilization tested after different storage durations (0, 5, 8 and 11 days after the end of the immobilization): $\alpha_{\text{AcPh}}=0.21$, $\alpha_{\text{w}}=0.50$, $n_{2\text{-prop}}/n_{\text{AcPh}}=60$, $m=50\text{ mg}$, $V_{\text{tot}}=20\text{ mL/min}$, $T=40^\circ\text{C}$.

The observed phenomenon was initially attributed to the water content of the immobilized preparations. Nevertheless offline measurement of the water content of the samples, by means of Karl-Fischer titration, revealed that the immobilized preparation water content was initially (at the first opening of the vessel) slightly increased (approximately 0.34%) compared to the water content of the samples that were extracted after several openings of the vessel (approximately 0.30%). The results from the water content measurement could not explain the huge reaction rate variation measured in the gas/solid reactor.

A macroscopic inspection of the enzyme preparation samples gave a more plausible explanation for the online behavior. After several openings of one enzyme preparation vessel, thorough mixing and withdrawing of samples, the remaining deposited enzyme preparation looked different: the carriers with the deposited enzyme were free-flowing and behaved rather like a powder, while a freshly immobilized sample behaved like a sticky mass. This observation led to the conclusion that mixing of the deposited enzyme preparation and several samplings from it led to an alteration of the preparation due to mechanical stress during the repeated mixing. Probably the deposited enzyme was mechanically partly removed from the surface of the glass carriers and was distributed as powder between the carriers. As a result the samples introduced in the reactor after some openings of the vessel were a mixture of enzyme deposited on carriers and free enzyme powder.

The free enzyme powder might have been responsible for the increased initial specific reaction rate observed by the older samples, due to higher amounts of enzyme without carriers introduced in the reactor. Moreover, increased hydration of the enzyme powder is expected, compared to the distributed on the carriers' surface enzyme. On the contrary, though, this fraction of free enzyme might have been for the same reason much more labile, resulting in an enhanced first phase deactivation. It must be stressed, though, that this theory can only qualitatively explain the observed behavior. The decisive for the reaction progress parameter, namely the fraction of the free enzyme in the sample introduced in the reactor was not controlled and, therefore, the resulting reaction and deactivation rate could not be predicted or reproduced. The typically large errors resulting from handling of solids emerged.

Supporting to this theory was the observation that immobilized samples that were stored for some time without being opened and mixed, gave results comparable to those obtained by freshly immobilized enzyme preparations. Therefore, the decisive factor to be controlled was not the storage duration but the handling of the enzyme preparation. As a result, the stability studies were performed with freshly immobilized enzyme preparations, in order to rule out the aforementioned effect.

When working with freshly immobilized enzyme preparations, though, the reproducibility of the measurements was again distorted, this time due to the difficulty to homogeneously mix the initially sticky mass. The extracted samples to be introduced in the reactor were thus not always representative of the specific activity (in IU/mg_{carriers}) of the whole preparation.

A simple way to increase the mechanical stability of the deposited preparation and avoid the leaching of the enzyme from the carriers` surface is offered by the use of sucrose as additive during the immobilization process. The beneficial effect of sucrose with respect to stability is extended through the whole process, starting from the enzyme deposition procedure, to the storage of the deposited enzyme and finally to its use in the gas/solid reactor.

More specifically, during the deposition process, in the presence of sucrose, the enzyme retained reproducibly almost 90% of its initial activity, while in the absence of sucrose only 60-70% was retained. Additionally, the storage stability of the deposited enzyme without sucrose was low, at 4°C, without controlled water activity, with half life time of approximately 10 days, as it is indicated in Fig.4.24. After the deposition process that lasted 3 days the initial residual activity of the deposited preparation was determined. The subsequent measurements were normalized to this value. On the contrary, the half life time of deposited enzyme with sucrose exceeded 30 days (data not shown).

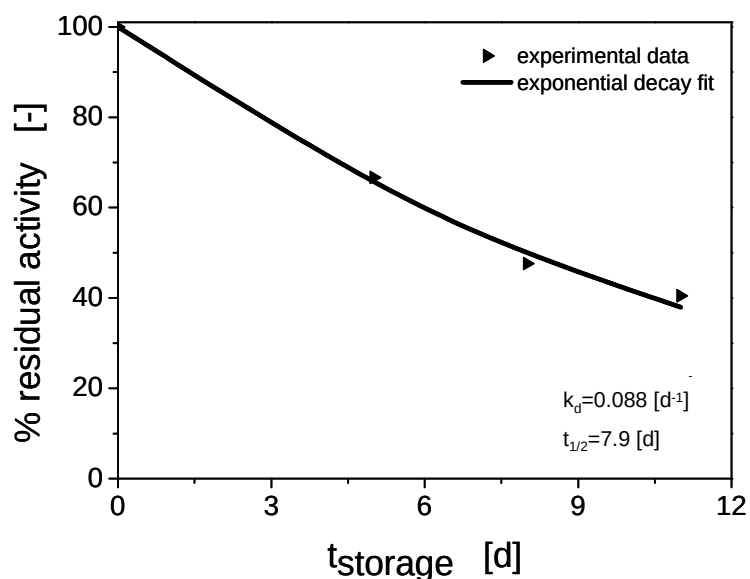


Figure 4.24 Storage stability of lyophilized wild type LBADH without sucrose, stored at 4°C.

The sucrose as an additive in the deposited enzyme preparation could also act as a stabilizer and reinforce the attachment of the enzyme to the surface of the carriers. Therefore, the mechanical stability of the deposited enzyme was higher.

Finally the positive effect of sucrose on the operational stability of the deposited enzyme preparation is elucidated in the following figure. The lyophilized wild type LBADH was deposited once without sucrose and once together with sucrose in a ratio $5 \text{ mg}_{\text{sucrose}}/\text{mg}_{\text{protein}}$. The two enzyme preparations were tested in the reactor under the same reaction conditions.

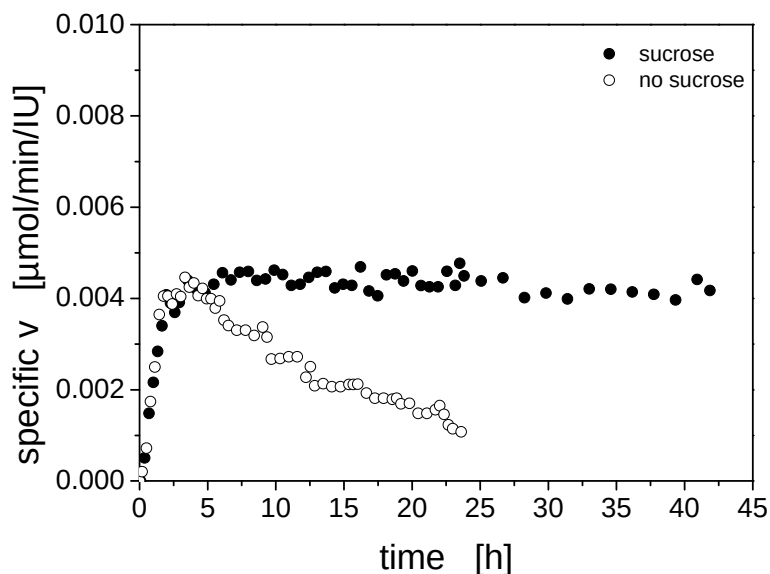


Figure 4.25 Effect of sucrose presence on the operational stability of lyophilized wild type LBADH: $\alpha_{AcPh}=0.35$, $\alpha_w=0.50$, $n_{2-prop}/n_{AcPh}=67$, $E_0=20.4$ IU, $V_{tot}=13$ mL/min, $T=40^\circ\text{C}$.

The enzyme preparation without sucrose appeared labile in the reactor. Within 22.4 hours, the reaction rate was decreased to the half of its initial value. The progress of the reaction of the deposited enzyme with sucrose on the other hand at the same reaction conditions was followed for approximately 45 hours, during which no significant deactivation was observed. The reaction was then interrupted since the operational stability of deposited LBADH preparations containing additives has been previously studied [Trivedi et al., 2006] and was beyond the scope of the present work. The order of magnitude of the half life time of the deposited enzyme preparation with sucrose under similar operating conditions was reported to be at around 1000 hours.

For the stability studies presented here, a sensitive system was required, that would allow fast observation of the deactivation, already within few hours. Thus the following studies were performed with deposited preparations without sucrose.

The operational stability of the deposited lyophilized wild type LBADH was compared to the stability of deposited cell extract of wild type LBADH in order to elucidate the role of the cell extract components presence. Storage stability studies, performed at an environment of controlled water activity and temperature,

indicated increased stability of the cell extract. The expectation was that the same stabilizing effect would be present also under operating conditions.

However, the operational stability of the cell extract was proven to be a lot lower compared to that of the lyophilized enzyme. More specifically the half life time of the cell extract was found to be less than one third of the half life of the lyophilized enzyme (6.6 hours vs. 22.4 hours).

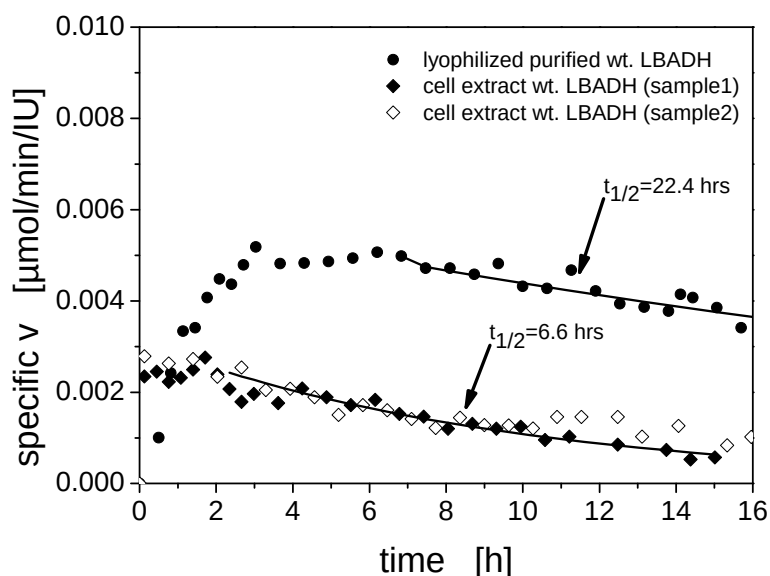


Figure 4.26 Operational stability of lyophilized and cell extract wild type LBADH: $\alpha_{AcPh}=0.35$, $\alpha_w=0.50$, $n_{2-prop}/n_{AcPh}=67$, $E_0=16$ IU, $V_{tot}=13$ mL/min, $T=40^\circ\text{C}$.

Obviously the deactivation mechanism of the reacting enzyme, being in the environment of substrates, is totally different to the deactivation mechanism under storage conditions where only water vapors are present. The components present in the cell extract seem to protect the enzyme from pure thermal denaturation. On the contrary, these components, in the presence of an organic substrate may probably tend to aggregate and in this way also affect the activity of the reacting enzyme.

It must be stressed, though, that the lyophilized enzyme did not originate from the same fermentation as the cell extract (purchased from Codexis). Significant differences, with respect to stability and kinetic parameters, between the same enzymes produced by different fermentation batches have been often reported

(apl. Prof. M. Pohl, personal communication) and as a result the specific compounds present in each cell extract may act differently, leading to either stabilization or destabilization.

4.5.2. Reaction conditions related parameters

The influence of the reaction conditions on the operational stability of the deposited enzyme was investigated. For this purpose, the water activity of the reaction mixture, the substrate activity and the amount of deposited enzyme preparation were individually varied and their influence on the stability was studied. Moreover, the study aimed at comparing the wild type LBADH and variant, G37D LBADH with respect to their performance in the gas/solid reactor.

The water activity influence was studied by introducing freshly immobilized samples into the reactor and acquiring their progress curve, keeping all reaction conditions constant apart from the water activity of the gas mixture. The water activity was varied within the range of 0.4 and 0.6 and the enzyme preparation stability was expressed as half life time.

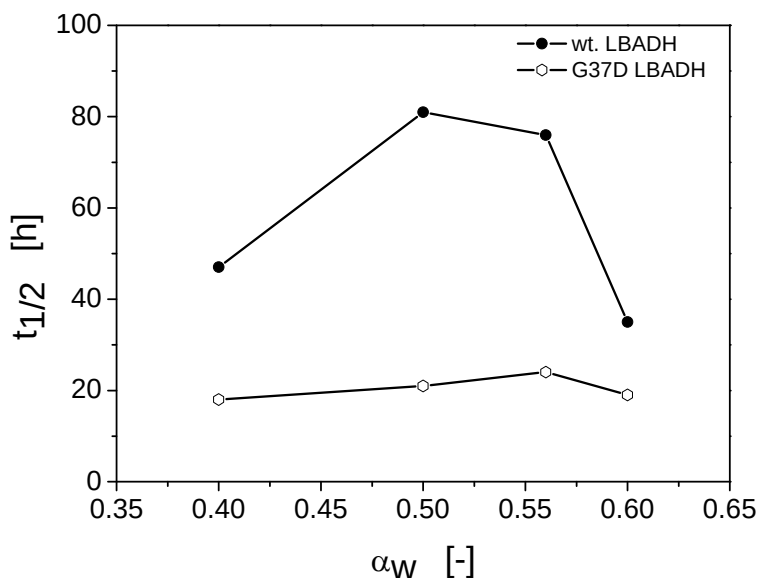


Figure 4.27 Influence of water activity on the operational stability of the wild type LBADH and the variant G37D LBADH: $\alpha_{AcPh}=0.3$, $n_{2-prop}/n_{AcPh}=60$, $m= 50$ mg (for wild type), $m=100$ mg (for lyophilized G37D), $V_{tot}=20$ mL/min, $T=40^{\circ}\text{C}$.

As it is depicted in Fig.4.27, the half life time of the purified and lyophilized deposited wild type LBADH exhibited a maximum at water activity around 0.50. This result contradicts previous findings [Trivedi, 2005], indicating a continuously decreasing stability of the deposited enzyme in the gas/solid reactor with increasing water activity but is in line with studies [Mikolajek et al., 2007] revealing an optimal water activity as here. A potential reason for the different behavior of the same enzyme might be the different processing of the enzyme prior to its deposition: here the used enzyme has been purified and lyophilized whereas the enzyme used in previous studies was a partly purified and lyophilized cell extract. The stability of the deposited lyophilized G37D variant was almost independent from the water activity.

The wild type LBADH appeared as the most stable of the two enzyme preparations, whereas the variant demonstrated a very low operational stability, with a half life time of around 22 hours for the whole range of water activities tested. The operational stability of the two enzymes was opposite to the expected behavior based on offline stability studies of the two enzymes in solution. The offline studies (L. Kulishova, ongoing thesis work) had indicated a much higher thermostability of the variant G37D through a range of temperatures between 30° and 70°C. At 40°C in particular the variant displayed an almost 20 times increased half life time compared to the wild type. Obviously, the stability in the gas/solid system is a property that can not be predicted by the stability in solution.

With respect to operational stability the wild type was, therefore, regarded as the best option, demonstrating the highest half life time at all water activities tested. Nevertheless, the reaction rates achieved by the two enzymes reversed this result. The less active lyophilized variant (4.5 IU/mg_{protein}), when tested in solution by the typical activity assay, demonstrated an unexpectedly high activity in the gas/solid reactor. This fact in combination with the much cheaper cofactor (NADH) required by the variant could possibly compensate the reduced stability of the variant in the gas/solid system.

To study the influence of the substrate (acetophenone) thermodynamic activity, the water activity was fixed at the value of 0.5, which was the water activity level at

which maximal stability was observed. The acetophenone activity was then varied in the range of 0.14 to 0.3, simultaneously varying the 2-propanol activity, thus keeping the molar ratio of the two substrates constant. The influence of the substrate activity on the stability of the deposited lyophilized variant and wild type was monitored and expressed by means of the half life time.

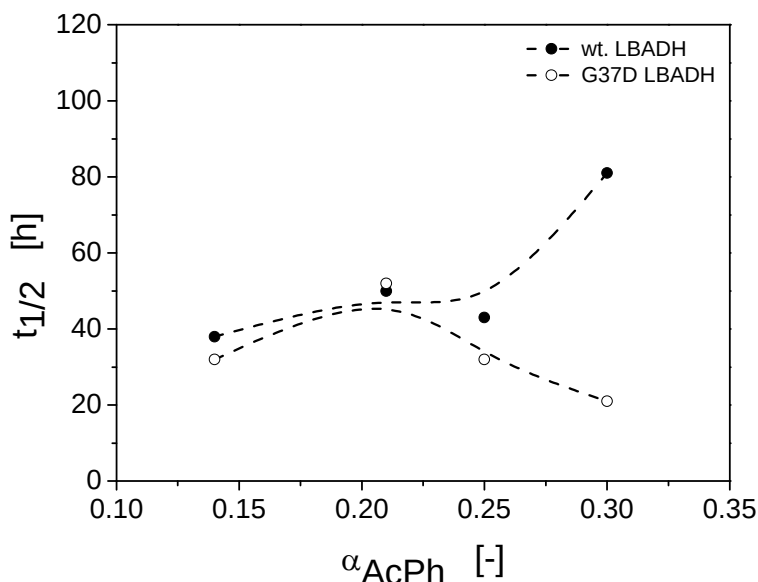


Figure 4.28 Influence of acetophenone thermodynamic activity on the operational stability of the deposited lyophilized wild type LBADH and variant G37D LBADH: $\alpha_w=0.50$, $n_{2-prop}/n_{AcPh}=60$, $m= 50$ mg (for wild type), $m=100$ mg (for lyophilized G37D), $V_{tot}=20$ mL/min, $T=40^\circ\text{C}$.

For the wild type LBADH, it was observed that the increase of the substrate activity, within the tested range, led to a stabilization of the enzyme. An increasing trend was revealed. The observed phenomenon could be possibly related to the competition for adsorption between water and substrates that might lead to stripping water off the enzyme and, therefore, a different deactivation route. In order to interpret this result, the influence of the substrate activity should be further investigated, at different water activity levels and probably also at varying 2-propanol to acetophenone molar ratios.

The stability of the lyophilized deposited variant G37D LBADH on the contrary revealed a different pattern, within the same range of acetophenone activity. The stability was increased by increasing the acetophenone activity from 0.14 to 0.21 and then decreased. The unexpectedly different influence of acetophenone activity

on the stability of the wild type and the variant is hard to interpret, due to the high structural similarity of the two enzymes. Further investigation is required in order to elucidate the destabilizing mechanism of acetophenone in the case of the variant. Since the stability studies for the investigation of the water and substrate thermodynamic activity influence were performed using different amounts of deposited enzyme preparation for the wild type and the variant (50 mg versus 100 mg), it was essential to investigate whether the enzyme amount influenced the operational stability and thus verify the comparability of the results.

Therefore, the potential influence of the enzyme amount on the operational stability was investigated by testing different amounts of deposited lyophilized G37D LBADH at a fixed set of conditions. The amount of enzyme preparation was varied in the range of 50 to 400 mg. The conversion in all cases was below 4% and thus the reactor was operated always at differential mode. This conclusion was strengthened by the constant initial specific reaction rate achieved at all amounts of enzyme preparation and the proportionality between the conversion and the enzyme amount (Fig.4.29 A and B).

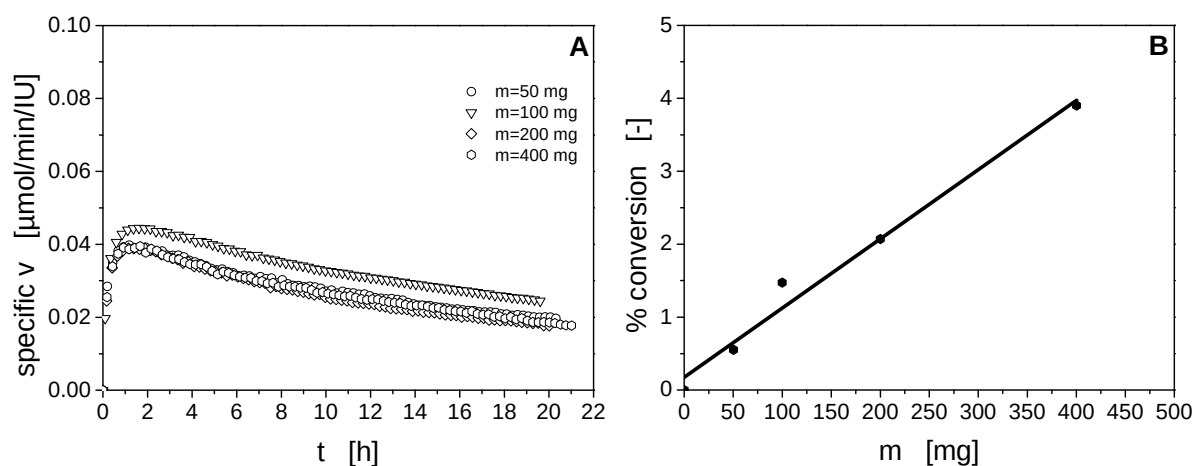


Figure 4.29 Influence of enzyme amount (bed length) on the specific reaction rate and conversion of the lyophilized mutant G37D LBADH: $\alpha_{\text{AcPh}}=0.3$, $\alpha_w=0.56$, $n_{2\text{-prop}}/n_{\text{AcPh}}=60$, $V_{\text{tot}}=20 \text{ mL}/\text{min}$, $T=40^\circ\text{C}$.

As it is shown in Fig.4.30 the amount of enzyme preparation did not influence the operational stability of the deposited enzyme. The half life time was constant at around 22 hours. On the contrary, previous stability studies performed with

carboligating enzymes [Mikolajek et al., 2007] indicated a dependency of the operational stability on the enzyme amount used in the gas/solid reactor.

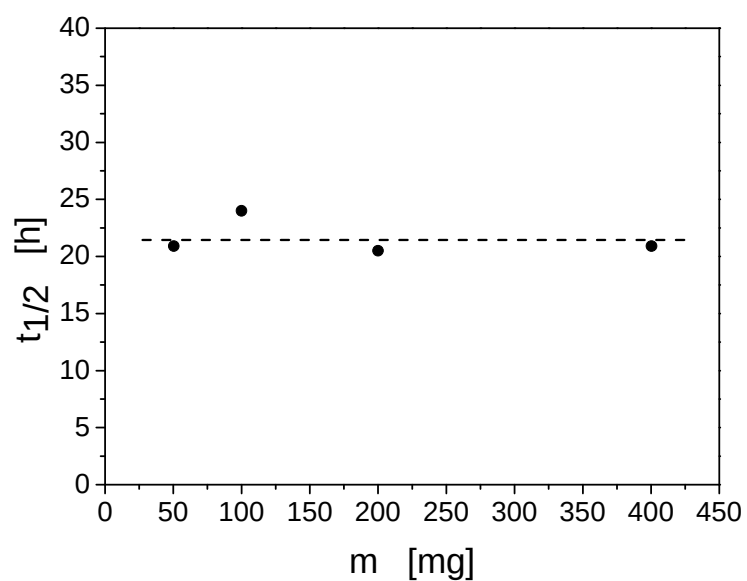


Figure 4.30 Influence of enzyme amount (bed length) on the operational stability of the lyophilized mutant G37D LBADH: $\alpha_{AcPh}=0.3$, $\alpha_w=0.56$, $n_{2-prop}/n_{AcPh}=60$, $V_{tot}=20$ mL/min, $T=40^\circ\text{C}$.

4.6. Enantioselectivity studies

The influence of the gas/solid reactor operation conditions on the enantioselectivity of the main reaction, namely the conversion of acetophenone to 1-phenylethanol was investigated. For this purpose, deposited cell extract, with and without sucrose was used. The parameters, expected to influence the enantioselectivity [Léonard et al., 2007], namely water activity of the reaction mixture, reaction temperature and acetophenone activity, were tested. Additionally, the enzyme amount and the presence of sucrose were also investigated. In order for a comparison to be plausible, the influence of each one of the aforementioned parameters was investigated, by keeping all other parameters constant. The varying conversion level (product amount) under the tested conditions might have also influenced the enantioselectivity, though. Nevertheless, this parameter can not be adjusted and was, therefore, investigated through the enzyme amount variation experiments. The measurement of the enantioselectivity was performed offline (section 3.4.3) and the enantioselectivity of the enzyme was estimated through the enantiomeric excess of the reaction mixture collected, throughout the whole reaction course.

4.6.1. Influence of enzyme amount

Previous studies (unpublished data) have indicated that the product amount might influence the enantioselectivity. Therefore, the effect of the varied parameters (water activity, substrate activity and temperature), tested in the following paragraphs, might be superimposed by the effect of the varying conversion, namely the varying amount of 1-phenylethanol. In order to isolate this influence, the amount of deposited enzyme preparation packed in the reactor was varied (from 50 to 600 mg) and the enantioselectivity of the reaction was measured at fixed reaction conditions. In this way, the water activity, substrate activity and temperature influences were ruled out.

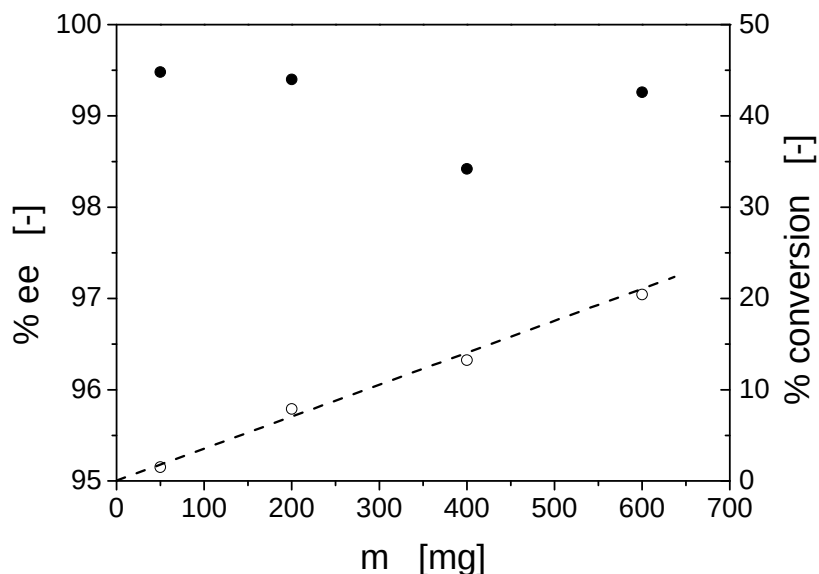


Figure 4.31 Influence of the amount of the deposited enzyme preparation on the enantioselectivity (closed symbols) and conversion (open symbols): $\alpha_w=0.46$, $\alpha_{AcPh}=0.21$, $n_{2-prop}/n_{AcPh}=72.4$, $E_0=60$ IU/g, $V_{tot}=19.3$ mL/min, $T=40^\circ\text{C}$.

It was shown (Fig.4.31) that the amount of deposited enzyme preparation used had no influence on the enantioselectivity. The enantiomeric ratio achieved remained around 99.4% while increasing the amount of enzyme preparation from 50 to 600 mg. A slightly lower enantioselectivity level was measured at 400 mg of deposited enzyme preparation; this was attributed though to measurement error. Throughout the whole range of deposited enzyme preparation, the reached conversion remained proportional to the catalyst amount.

This result indicated that the increased product amount did not tune the enantioselectivity of the LBADH, as previously measured (unpublished data) for the ADH T catalyzing the conversion of 2-butanone to 2-butanol. It is believed that the small product molecule in the latter case acts as a plasticizer to the enzyme active center, enabling in this way the formation of both enantiomers and thus leading to a decreased enzyme enantioselectivity. In contrast, the bulky 1-phenylethanol molecule probably does not act in a similar way and thus does not influence the enantioselectivity of the LBADH.

Since the product amount did not have an effect on the enantioselectivity, the remaining parameters could be studied, without taking into account the conversion level reached in each case. The initial specific activity (in IU/g) of the enzyme preparation used was not necessarily kept constant through the whole range of investigations, while the amount of enzyme preparation used was always kept constant at 400 mg. The resulting varying overall amount of enzyme activity units introduced in the reactor will have influenced the conversion but not the enantioselectivity, as previously shown.

4.6.2. Influence of water activity

The investigation of the water activity influence on the enantioselectivity of the reaction was performed by scanning the water activity of the reaction mixture in the range of 0.35 to 0.65. For each water activity, a new sample of enzyme preparation was introduced in the reactor and the reaction was initiated. Each reaction was performed for approximately 20 hours. The steady state conversion was documented and the enantioselectivity was determined through the enantiomeric excess.

As it is indicated by the following figure, the enantiomeric excess of the gas/solid conversion of acetophenone to 1-(*R*)-phenylethanol was above 96 % for all water activity levels tested. Nevertheless, the enantiomeric excess at low water activities is considered relatively low, when compared to the almost 100% values previously reported in liquid systems [Hildebrand and Lütz, 2006; Ferloni, 2004]. It increased though by increasing the water activity. At water activities above 0.55, where an elevated conversion due to the higher enzyme hydration was achieved, the enantiomeric excess reached a plateau at 99.5% and, therefore, nearly pure 1-(*R*)-phenylethanol was produced.

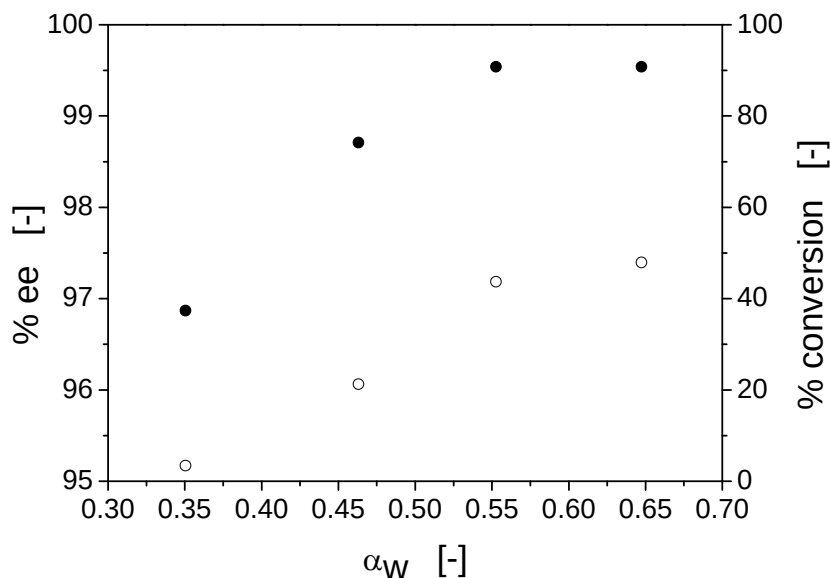


Figure 4.32 Dependency of the enantioselectivity (closed symbols) and conversion (open symbols) on the water thermodynamic activity of the reaction mixture: $\alpha_{AcPh}=0.3$, $n_{2-prop}/n_{AcPh}=50$, $m=400$ mg, $E_0=250$ IU/g, $V_{tot}=19.3$ mL/min, $T=40^\circ\text{C}$.

The increase of the enantioselectivity, at elevated water activities, could be attributed to the higher enzyme flexibility through the increased hydration. The water activity does not only influence the enzyme activity in a positive manner, by conferring to the enzyme its catalytically active conformation, but also its stereospecificity by possible interactions with the active site [Léonard et al., 2007]. The exact mechanism can only be elucidated by means of molecular modeling and is far beyond the scope of the present work.

The effect of the thermodynamic water activity on the enantioselectivity of enzymes has been widely studied, mainly in organic media. The outcome of these studies did not give a clear trend of the influence, though. There have been cases reported where the increase of the water activity had a positive effect on the enantioselectivity [Jönsson et al., 1999; Persson et al., 2002], a negative one [Persson et al., 2002; Ducret et al., 1998] or no effect at all [Persson et al., 2002; Wehtje et al., 1997].

4.6.3. Influence of temperature

At a fixed water activity of 0.55, where the enantiomeric excess achieved in the gas/solid system was >99.5% the influence of the reaction temperature was investigated. The other parameters were kept constant. The further temperature levels tested were 45°C, 50°C and 60°C.

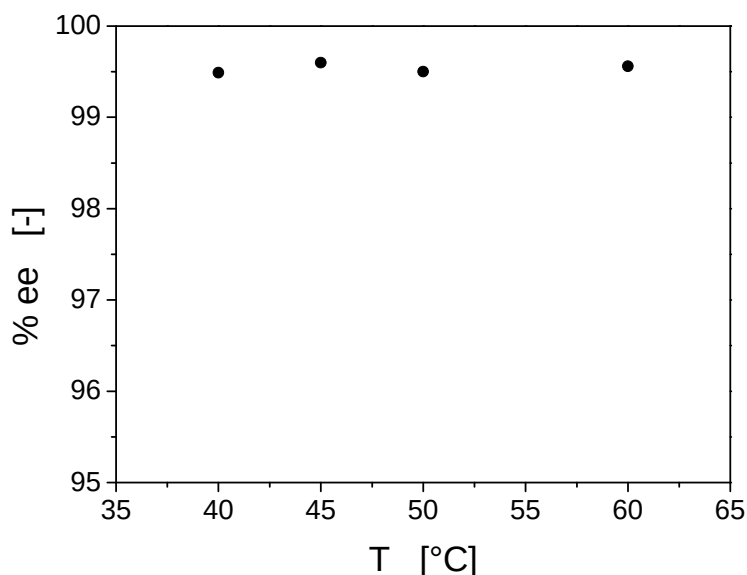


Figure 4.33 Dependency of the enantioselectivity on the operation temperature: $\alpha_w=0.55$, $\alpha_{AcPh}=0.3$, $n_{2-prop}/n_{AcPh}=50$, $m=400$ mg, $E_0=160$ IU/g, $V_{tot}=19.3$ mL/min.

As it is shown in Fig.4.33, the reaction temperature did not influence the enantioselectivity of the reaction. The enantiomeric excess remained >99.5% fluctuating slightly, with no clear trend though.

It has been previously reported [Yang et al., 1997] that the stereoselectivity of alcohol dehydrogenases in organic or aqueous media depends on the reaction temperature. In some cases the enantiomeric excess of the product increases, in other cases decreases while there are cases where the enantiomeric excess is almost not influenced by increasing temperature or the effect is hard to determine due to the very high enantiomeric excess achieved at all temperature levels studied, like in the present study.

The temperature effects on the enantioselectivity of alcohol dehydrogenases catalyzing the oxidation of chiral alcohols have been previously investigated [Philips, 1996] using thermodynamic and kinetic data, whereas it has been also shown that the reduction of the prochiral ketone can be treated in a similar way [Yang et al., 1997].

In the present study the aforementioned prediction process could not be applied, due to lack of kinetic and thermodynamic data. The determination of the enantioselectivity was based on the enantiomeric excess of the product collected through the whole reaction. The progress curves of the two enantiomers could not be acquired due to the very low amount of the *S* enantiomer and, therefore, the respective initial rates could not be determined.. Moreover the prerequisite for kinetic control and substrate concentration far below the K_m value was not fulfilled.

4.6.4. Influence of acetophenone activity

The influence of acetophenone thermodynamic activity on the enantioselectivity in the gas/solid reactor was investigated at fixed temperature (40°C) and fixed 2-propanol activity (0.174). The fixed water activity level of the reaction mixture for this investigation was selected to be 0.46. At this water activity level the enantiomeric excess was slightly lower, below 99% (section 4.6.2), leading to a higher amount of produced 1-(*S*)-phenylethanol and, therefore, possible changes in the enantioselectivity, due to the variation of the substrate activity, would be more easily detected.

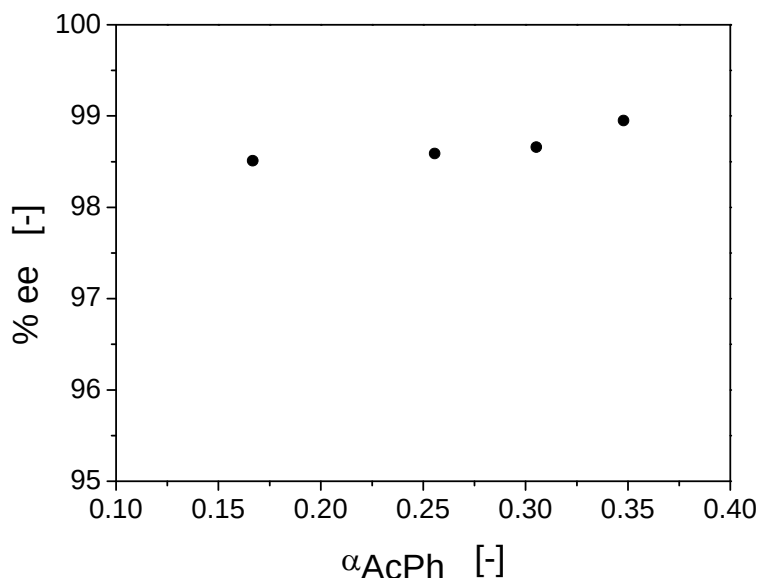


Figure 4.34 Dependency of the enantioselectivity on the acetophenone thermodynamic activity: $\alpha_w=0.46$, $\alpha_{2\text{-prop}}=0.174$, $m=400$ mg, $E_0=208$ IU/g, $V_{\text{tot}}=19.3$ mL/min, $T=40^\circ\text{C}$.

The increase of the thermodynamic activity of acetophenone led to a minor increase in the enantioselectivity (from 98.5% at acetophenone activity 0.15 to 99% at acetophenone activity 0.35).

By keeping the activity of the co-substrate, 2-propanol, constant at all acetophenone activity levels, the previously studied [Yang et al., 1997] co-substrate effect was avoided. Nevertheless, the resulting varying ratio of 2-propanol to acetophenone changed the equilibrium position and this might have possibly influenced the selectivity.

4.6.5. Influence of sucrose presence

The potential influence of the sucrose, used during the enzyme immobilization process, on the enantioselectivity of the deposited enzyme preparation was investigated. For this purpose, a second batch of deposited enzyme was prepared, without the co-deposition of sucrose.

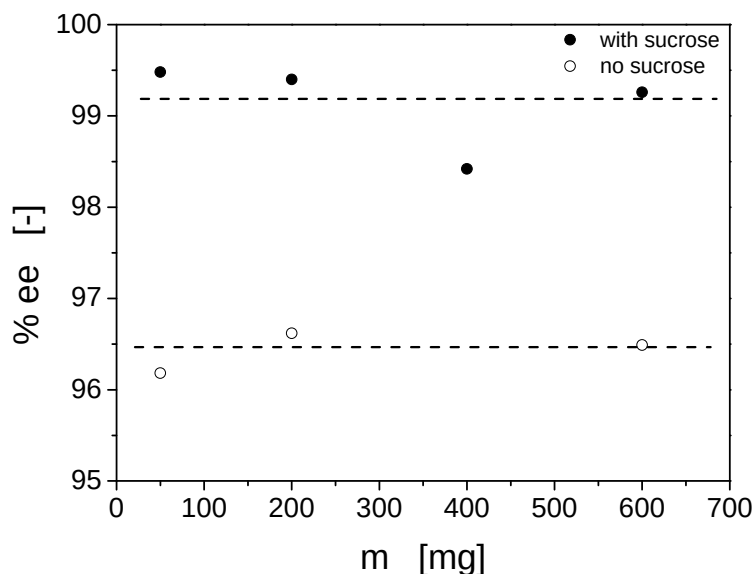


Figure 4.35 Influence of sucrose presence on the enantioselectivity of the deposited enzyme preparation: $\alpha_w=0.46$, $\alpha_{AcPh}=0.21$, $n_{2-prop}/n_{AcPh}=72.4$, $E_0=60$ IU/g, $V_{tot}=19.3$ mL/min, $T=40^\circ\text{C}$.

As it is indicated in Fig.4.35, the presence of sucrose had a positive effect on the enantioselectivity. The deposited enzyme preparations without sucrose gave a constant, with respect to the enzyme preparation amount, enantiomeric excess of around 96%, significantly lower thus, compared to the enantiomeric excess of the preparations with sucrose (around 99%).

The most probable explanation of the observed beneficial effect of sucrose is connected to the different enzyme hydration at the presence and absence of sucrose, described in section 4.2.3. The amount of water adsorbed by the deposited enzyme preparation without sucrose is lower compared to that adsorbed in the case of sucrose being present. Therefore, the observed lower enantioselectivity in the former case is most probably connected to the lower enzyme hydration. The beneficial role of the increased water availability for the LBADH enantioselectivity has already been depicted in section 4.6.2.

Nevertheless, in order to exclude any potential structural enzyme changes, triggered by the co-deposition with sucrose, which might have led to enhanced enantioselectivity, a series of enantioselectivity studies with deposited enzyme preparations without sucrose, at different water activity levels must be performed.

5. Conclusions and Outlook

The present project demonstrated the construction and characterization of a continuous gas/solid reactor and the description of the overlaying thermodynamic and kinetic phenomena taking place during the reduction of acetophenone to 1-(*R*)-phenylethanol with the concomitant oxidation of 2-propanol to acetone catalyzed by deposited LBADH. The role of water was proven to be central influencing and interconnecting the separately studied phenomena. Finally, the immobilization method by means of adsorption to non-porous carriers employed in this project was challenged.

The first major task of this project was the construction of a new automated continuous gas/solid reactor and the validation of its operation. The reactor set-up as well as the integrated analytical device (GC) were calibrated and tested in order to verify the ability to thermodynamically control the system, according to the theoretical equilibrium calculations. It was accordingly proven that the thermodynamic control was efficient within the system's operation window. The equilibration of the carrier gas in the saturation unit with the liquid substrates as well as the control of their thermodynamic activity throughout the entire reactor set-up was efficient.

In order to achieve this control, efficient heating of the reaction mixture was essential. In the present form of the reactor set-up efficient tempering of the reaction mixture through the entire set-up required extended tubing, acting as passive heating spirals, for the gaseous reaction mixture. Although the target of efficient tempering was achieved, adsorption of the substrates to the inner surface

of the tubing was present leading to long equilibration times and very slow response of the system. Therefore, a future upgrading and optimization of the set-up by replacing the heating coils through thermocouples, actively heating the tubing, is essential. The reactor size would be in this way drastically minimized so that the equilibration of the reaction mixture would take place in a significantly shorter time, enabling in this way the faster equilibration of new conditions and performance of more experiments. The replacement of the stainless steel material through a different, passivated steel quality that would only minimally interact with the reaction mixture components should be also considered.

Another critical point at the present set-up is the tubing part connecting the two units which is exposed to the environment. Although the currently used external active tempering of this part, by means of a water-bath, was effective, its length should be in the future also minimized, in order for the heat loss to the environment to be as low as possible. This would also allow the operation of the reactor at elevated temperatures without the risk of condensation of the gas mixture components within this part.

Although the control of the thermodynamic activities of the reacting compounds was possible at the present set-up by means of the GC, the humidity of the reaction mixture remained the 'black box' of the system. In the future, a validation of the system with respect to the humidity of the reaction mixtures formed should be performed by integrating a humidity sensor in the system and controlling the achieved humidity in the absence of the reaction mixture compounds vapors. By means of the humidity sensor the unexpected condensation taking place at humidity levels above 70% could be also elucidated.

During the reactor characterization, axial dispersion of the reacting compounds along the packed-bed was predicted. Nevertheless, due to the short length of the reactor packed-bed, the dispersion leading to delayed response of only seconds at the reactor outlet could not be experimentally verified. The respective step experiments revealed, though, that the dispersion over the packed-bed was overlaid by a pronounced adsorption of acetophenone to the packed bed.

The adsorption of the two substrates of LBADH, acetophenone and 2-propanol, was, therefore, investigated for the first time, at conditions mimicking those of a reacting system, allowing a more complete insight into the microenvironment of the deposited enzyme. It was revealed that 2-propanol does not get adsorbed at a level detectable at the specific set-up but acetophenone is adsorbed to the deposited enzyme preparation at a significant level. This might lead to limitations due to diffusional transport to the enzyme influencing in this way the reaction kinetics in a reacting system. Therefore, the adsorption studies need to be linked to kinetic studies in the future, in order to investigate the aforementioned effects.

Due to the central role of water in the gas/solid system its adsorption by the deposited enzyme preparation was thoroughly investigated at conditions resembling those of the gas/solid reactor. Therefore, a simple and efficient experimental set-up, the water adsorption unit, was constructed and employed in order to monitor the water adsorption to the deposited LBADH. The hydration of the deposited enzyme was described by a BET-like isotherm. The critical thermodynamic water activity level, above which the system deviates from the initial adsorption behavior, was found to be approximately 0.5. It was shown that the presence of sucrose in the enzyme preparation has an important influence on the capacity of the packed bed to adsorb and retain water. It was also shown that the hysteresis during water desorption from the deposited preparation is very significant. This is accompanied by micro-structural changes of the lyophilized or deposited enzyme preparations, leading to leaching of the enzyme from its support at higher humidity levels. This effect was the first indication suggesting the choice of a stronger, probably covalent immobilization method in the future.

The enzyme activity at different hydration levels was studied aiming at correlating the influence of the adsorbed amount of water on the reactivity of the enzyme and identifying the minimal water amount required by the deposited enzyme preparation in order for the enzyme to become active and give measurable reaction rates. It was, therefore, shown that, at the tested reaction conditions, the minimal water activity at which measurable conversion at the reactor was achieved was between 0.2 and 0.25, with a corresponding amount of approximately

5 mg_{water}/mg_{protein} of adsorbed water. By further increase of the water activity of the reaction mixture, an almost exponential increase of the conversion was monitored.

The kinetic investigation revealed that the two parallel reactions, coupled through the cofactor regeneration demonstrate a time shift, with the 2-propanol conversion preceding. This phenomenon was attributed to a faster equilibration of 2-propanol with the deposited enzyme compared to acetophenone, indicated also through the adsorption studies. Moreover, a first order kinetics with respect to acetophenone and a Michaelis-Menten pattern with respect to 2-propanol was revealed, during the kinetic investigation performed under strictly reaction rate limiting conditions. Pronounced diffusional limitation at high specific activities of deposited enzyme preparation with sucrose was demonstrated. This again indicated the weakness of the deposition method and the need for alternative immobilization methods, probably into porous carriers.

The stability studies under operating conditions indicated a pronounced influence of the water activity on the stability of the purified and lyophilized wild type LBADH that was deposited without sucrose. By increasing the water activity in the range of 0.4 to 0.6, the stability was initially increased reaching an optimum at around 0.5 and then decreased again. The purification state of the enzyme prior to its deposition should be in the future investigated giving an insight on its potential effects on the operational stability in the gas/solid reactor.

The comparison of the wild type with the purified and lyophilized deposited variant G37D, a promising candidate due to its dependency on NADH, instead of NADPH, and its enhanced offline stability, indicated that the increased stability of the latter measured in solution was not transferred in the gas/solid system and thus the variant was less stable than the wild type under the same operating conditions. It was, therefore, once more demonstrated that the prediction of enzyme properties from data obtained in solution is not straightforward.

One more important outcome from the stability studies without the use of sucrose as stabilizer was the low sustainability of the deposition method as a method of enzyme immobilization. The very weak attachment of the simply deposited and

dried enzyme to the non porous glass carriers led not only to leaching of the biocatalyst during operation at high water activity levels but also to an unstable preparation which was prone to mechanical stress and disruption of the enzyme from the carriers' surface. These findings also suggest the need for development of an alternative immobilization method in the future.

With respect to the enantioselectivity investigations, the acetophenone conversion was performed with high enantioselectivity in the gas/solid reactor. The enantiomeric excess achieved was always high approaching 99.5 %, at all commonly used operating conditions. The most significant parameter influencing the enantioselectivity was found to be the humidity of the reaction mixture. Additionally, a positive effect of the presence of sucrose was identified; it was attributed, though, to the different hydration of the enzyme in the presence and absence of sucrose. Although the increased enantioselectivity supports the performance of the acetophenone reduction at the gas/solid system it, nevertheless, renders this system inappropriate for investigating the enantioselectivity of the deposited LBADH due to its low sensitivity. In the future a different reaction system, like the reduction of 2-butanone to 2-butanol, demonstrating a decreased enantioselectivity, could be employed for investigating the enantioselectivity of the LBADH and elucidating the influencing parameters.

In the future, the current project should be further continued towards two main directions. The first one is connected to the optimization of the constructed reactor set-up in order to achieve its miniaturization and thus a reduced substrate adsorption and shorter response times. This modification is considered essential, in particular with respect to substrate compounds of low volatility, like acetophenone, and high affinity to the stainless steel inner surface of the pipeline, like phenylethanol.

The second direction is connected to the optimization of the immobilization process. The present work demonstrated the weaknesses of the currently used deposition process. The important role of the enzyme processing prior to its deposition for the catalyst operational stability and reproducibility of the measurements was also demonstrated. Significant differences between purified

enzyme and enzyme contained in cell extract, as well as a great effect of the lyophilization prior to deposition were indicated. Therefore, a systematic investigation of the effect of the immobilization conditions as well as the impact of the enzyme carriers should be performed in the future. The investigation should aim at identifying a simple immobilization process that would nevertheless yield catalysts with optimal activity, stability and selectivity in the gas/solid system.

In an optimized reactor set-up and with an optimally prepared catalyst a complete characterization of the parallel thermodynamic and kinetic phenomena occurring during catalysis should be performed. Final target should be the construction of an integrated model that will allow carrying out simulations able to predict the reactor performance as a function of the operation conditions.

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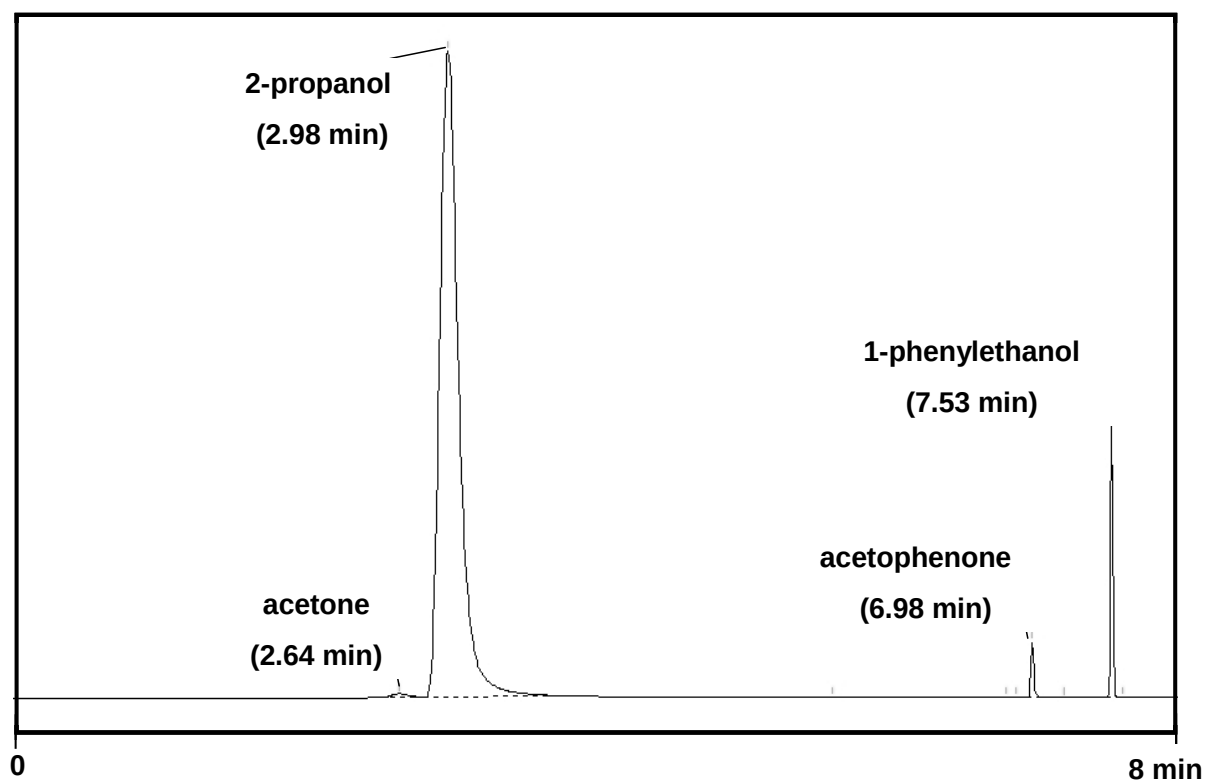
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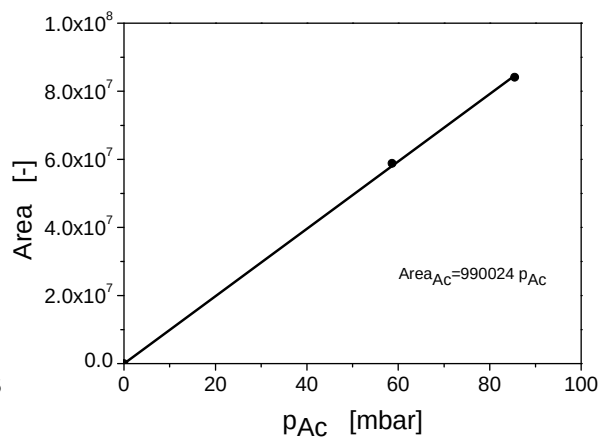
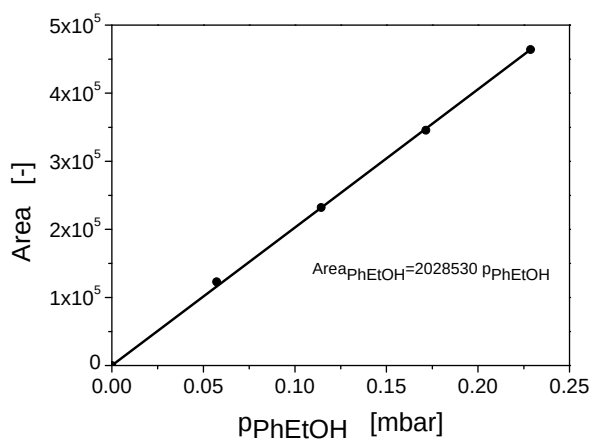
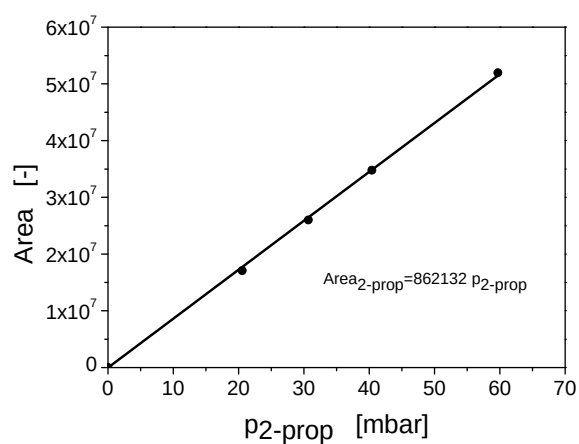
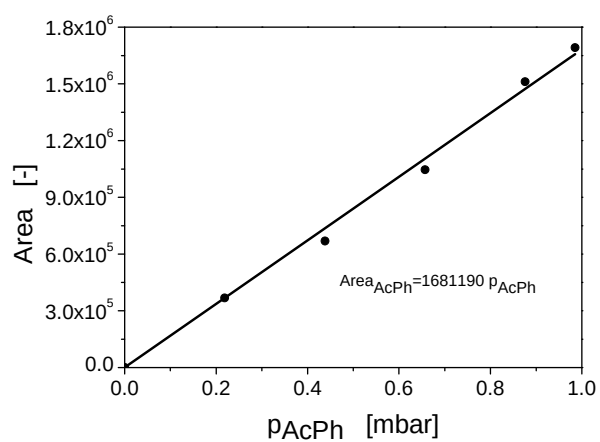
Appendix

Appendix A: Gas chromatography

Online reaction mixture separation



Online calibration of reaction components



Appendix B: Antoine parameters

Compound	A [-]	B [K]	C [K]	T _{range} [K]
Acetophenone	4.64896	2006.397	-43.472	310.2-475.5
2-propanol	4.86100	1357.427	-75.814	329.9-362.4
Phenylethanol	5.36689	2479.570	-30.510	331.3-492.6
Acetone	4.42448	1312.253	-32.445	259.2-507.6
Water	6.20963	2354.731	7.559	293-343

Source: NIST

Appendix C: gPROMS Simulation files

Saturation process

MODEL

PARAMETER

g	AS REAL	#gravity [m/s^2]
#Physical properties of water		
Diff	AS REAL	#diffusion coefficient of water in N2 [m^2/s]
s_t	AS REAL	#surface tension:water against air [Kg/s^2]
d	AS REAL	#density of water at T=45°C [Kg/m^3]
#Geometrical characteristics of bubbler		
D_h	AS REAL	#vent opening diameter [m]

DISTRIBUTION_DOMAIN

radial AS [0 : 1]

VARIABLE

p_w droplet	AS DISTRIBUTION(radial) OF p	#partial pressure of water in the
p_ws water	AS p	#saturation partial pressure of
P_s	AS DISTRIBUTION(radial) OF degree_s	#degree of saturation
r_0	AS r_0	

BOUNDARY

PARTIAL(p_w(0),radial)=0;
p_w(1) = p_ws;

EQUATION

#Bubble diameter calculation
r_0=(3*D_h*s_t/(4*g*d))^(1/3);

#Mass balance over the bubble
FOR r := 0|+ TO 1|- DO
\$p_w(r)*r=Diff/(r_0^2)*(r*Partial(p_w(r),radial,radial)+2*Partial(p_w(r),radial));
END

#Degree of saturation in the bubble
FOR r := 0 TO 1 DO
P_s(r)=p_w(r)/p_ws;
END

PROCESS

UNIT

bubbler_water AS bubbler_water

SET

WITHIN bubbler_water DO

g := 9.8; #gravity [m/s^2]

#Physical properties of water

Diff := 23.9E-6;

#diffusion coefficient of water in N2 [m^2/s]

s_t:= 0.068735;

#surface tension of water against air [Kg/s^2]

d := 990.2;

#density of water at T=45°C [Kg/m^3]

#Geometrical characteristics of bubbler

D_h := 0.0015; #gas outlet diameter [m]

radial := [CFDM, 2, 50];

END

ASSIGN

WITHIN bubbler_water DO

p_ws := 0.0948; #partial saturation pressure of water at T=45°C

END

INITIAL

WITHIN bubbler_water DO

FOR r := 0|+ TO 1|- DO

p_w(r) = 0;

END

END

SOLUTIONPARAMETERS

DASolver := "DASOLV"

REPORTINGINTERVAL :=0.01;

SCHEDULE

CONTINUE FOR 5;

Dispersion over the packed-bed

PULS MODEL

MODEL

PARAMETER

R	AS REAL	#gas constant
Pi	AS REAL	#pi=3.14
D_m	AS REAL	
Voidage	AS REAL	#voidage

#Antoine coefficient A of compound i

A	AS REAL
---	---------

#Antoine coefficient B of compound i

B	AS REAL
---	---------

#Antoine coefficient C of compound i

C	AS REAL
---	---------

#Geometrical parameters

radius, L	AS REAL
-----------	---------

DISTRIBUTION_DOMAIN

Axial	AS [0 : L]
-------	------------

VARIABLE

V_tot	AS flowrate	#total volumetric flowrate [L/h]
T	AS temperature	#reactor unit temperature [K]
p_s	AS pressure	#saturation pressure [bar]
therm_a	AS DISTRIBUTION(Axial) OF Therm_activity	#thermodynamic activities of
compounds [-]		
therm_a_in	AS Therm_activity	
v	AS velocity	
D_e	AS D_e	#effective diffusion coefficient

BOUNDARY

#BC1 at the reactor inlet

therm_a(0)=therm_a_in;

#BC2 at the reactor outlet

PARTIAL(therm_a(L), Axial) = 0;

EQUATION

#Interstitial velocity over the packed bed

$v = V_tot / (\pi * radius^2);$

#Effective dispersion coefficient

$D_e = D_m; \# + v^2 * radius / (48 * D_m);$

#Antoine equation - Calculation of saturation pressure in reaction unit

$p_s = 10^{(A - (B / (C + T)))};$

#Mass balances

FOR z := 0|+ TO L|- DO

$\$therm_a(z) * (1 - voidage) * \pi * radius^2 = -V_tot * PARTIAL(therm_a(z), Axial) + (1 - voidage) * \pi * radius^2 * D_e * PARTIAL(therm_a(z), Axial, Axial);$

END

PROCESS

UNIT

pulse AS pulse

SET

WITHIN pulse DO

R := 83.144E-6;

pi := 3.14;

A := 4.64896;

B := 2006.397;

C := -43.472;

radius := 0.0025;

L := 0.018;

voidage := 0.3;

D_m := 1E-5;

Axial := [CFDM, 2, 200];

END

gas constant - UNIT [m3 bar/mmol K]

#Antoine coefficient A for the acetophenone

#Antoine coefficient B for the acetophenone

#Antoine coefficient C for reactants and products

#reactor radius - UNIT [m]

#packed bed length - UNIT [m]

#voidage of the packed bed - UNIT [-]

#axial dispersion coefficient UNIT[m2/s]

ASSIGN

WITHIN pulse DO

V_tot := 0.233E-6;

UNIT [m3/s]

T := 303;

therm_a_in := 0;

END

#=14mL/min; total volumetric flow in the reactor -

#reaction temperature - UNIT [K]

#initial thermodynamic activity - UNIT [-]

INITIAL

WITHIN pulse DO

FOR z:= 0|+ TO L|- DO

therm_a(z) = therm_a_in;

END

END

SOLUTIONPARAMETERS

REPORTINGINTERVAL :=0.01;

SCHEDULE

SEQUENCE

CONTINUE FOR 1;

RESET

WITHIN pulse DO

therm_a_in := 0.15;

END

END

CONTINUE FOR 0.02;

RESET

WITHIN pulse DO

therm_a_in := 0;

END

END

CONTINUE FOR 10;

END

STEP MODEL

MODEL

PARAMETER

R	AS REAL	#gas constant
Pi	AS REAL	#pi=3.14
D_m	AS REAL	
Voidage	AS REAL	

#Antoine coefficient A of compound i

A	AS REAL
---	---------

#Antoine coefficient B of compound i

B	AS REAL
---	---------

#Antoine coefficient C of compound i

C	AS REAL
---	---------

#Geometrical parameters

radius, L	AS REAL
-----------	---------

DISTRIBUTION_DOMAIN

Axial	AS [0 : L]
-------	------------

VARIABLE

V_tot	AS flowrate	#total volumetric flowrate [L/h]
T	AS temperature	#reactor unit temperature [K]
p_s	AS pressure	#saturation pressure [bar]
therm_a	AS DISTRIBUTION(Axial) OF Therm_activity	#thermodynamic activities of
compounds [-]		
therm_a_in	AS Therm_activity	
v	AS velocity	
D_e	AS D_e	#effective diffusion coefficient

BOUNDARY

therm_a(0)=therm_a_in;

PARTIAL(therm_a(L), Axial) = 0;

EQUATION

#Interstitial velocity over the packed bed

$v = V_tot / (\pi * radius^2);$

#Effective dispersion coefficient

$D_e = D_m;$

#Antoine equation - Calculation of saturation pressure in reaction unit

$p_s = 10^{(A - (B / (C + T)))};$

#Mass balances

FOR z := 0|+ TO L|- DO

$\$therm_a(z) * (1 - voidage) * \pi * radius^2 = -V_tot * PARTIAL(therm_a(z), Axial) + (1 - voidage) * \pi * radius^2 * D_e * PARTIAL(therm_a(z), Axial, Axial);$

END

PROCESS

UNIT

stepp AS stepp

SET

WITHIN stepp DO

R := 83.144E-6;

pi := 3.14

A := 4.64896;

B := 2006.397;

C := -43.472;

radius := 0.0025;

L := 0.01;

voidage := 0.3;

D_m := 1E-5;

Axial := [CFDM, 2, 200];

END

gas constant - UNIT [m3 bar/mmol K]

#Antoine coefficient A for the acetophenone

#Antoine coefficient B for the acetophenone

#Antoine coefficient C for the reactants and products

#reactor radius - UNIT [m]

#packed bed length - UNIT [m]

#voidage of the packed bed - UNIT [-]

#axial dispersion coefficient UNIT[m2/s]

ASSIGN

WITHIN stepp DO

V_tot := 0.166E-6;

[m3/s]

T := 313;

therm_a_in := 0;

END

#=10mL/min; total volumetric flow in the reactor - UNIT

#reaction temperature - UNIT [K]

#initial thermodynamic activity - UNIT [-]

INITIAL

WITHIN stepp DO

FOR z:= 0|+ TO L|- DO

therm_a(z) = therm_a_in;

END

END

SOLUTIONPARAMETERS

REPORTINGINTERVAL :=0.01;

SCHEDULE

SEQUENCE

CONTINUE FOR 1;

RESET

WITHIN stepp DO

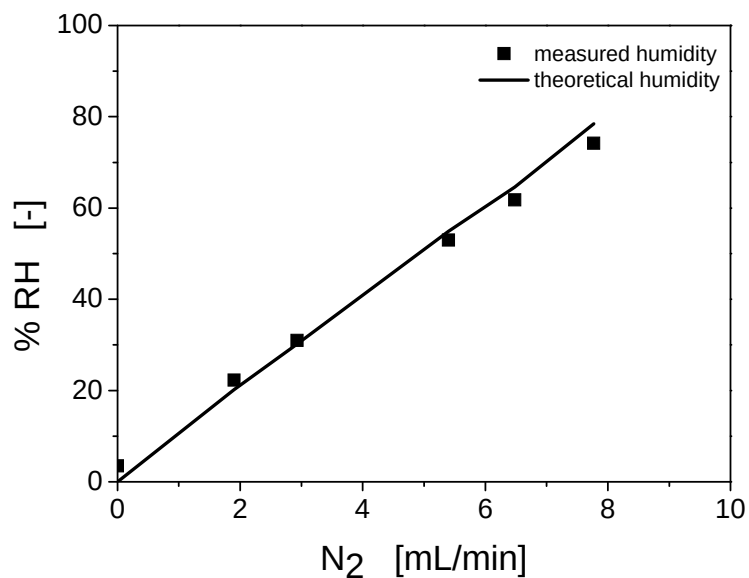
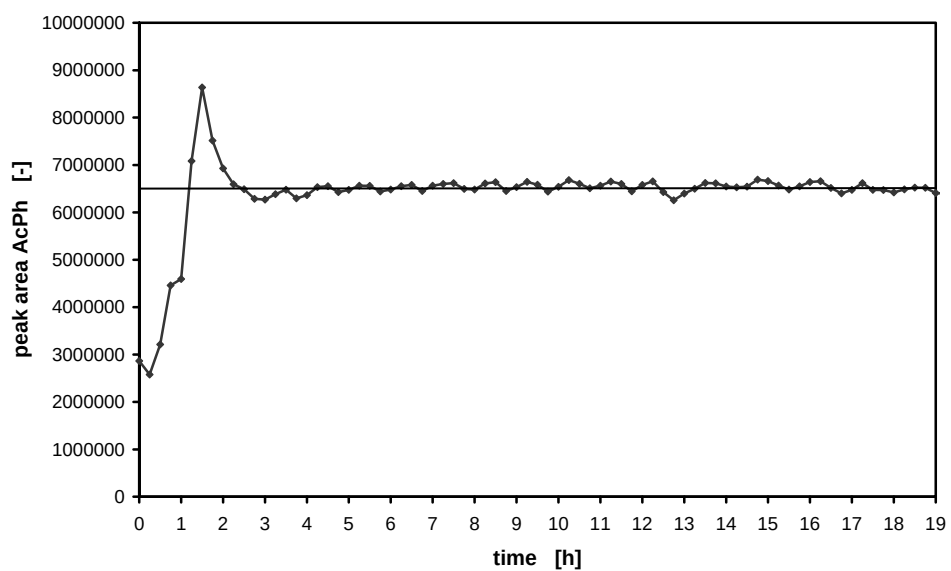
therm_a_in := 0.547;

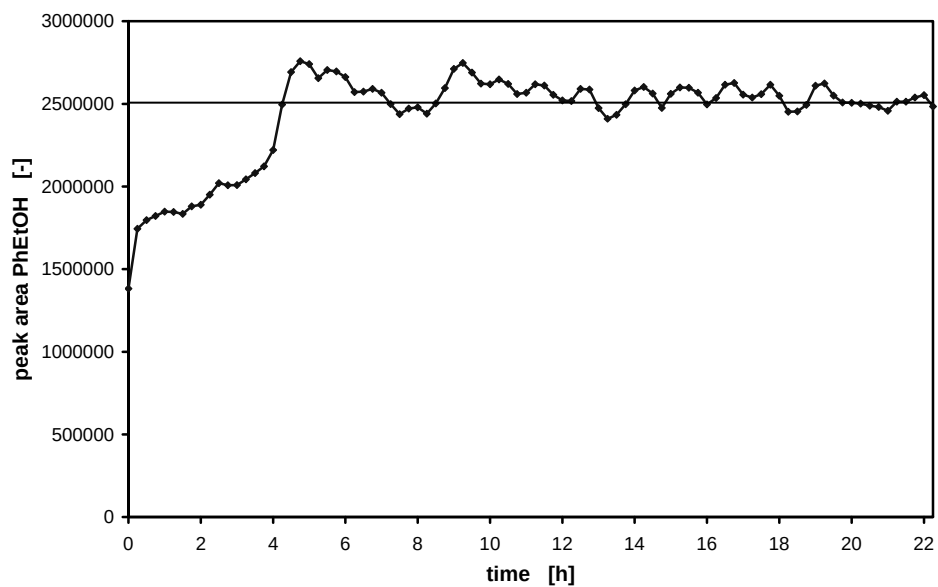
END

END

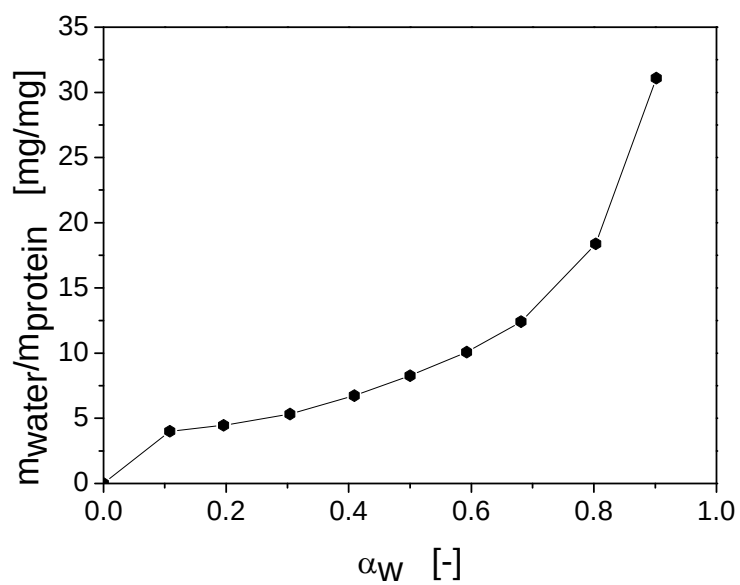
CONTINUE FOR 10;

END

Appendix D: Experimental data**Nitrogen saturation with water in the water adsorption unit****Acetophenone and 1-phenylethanol equilibration directly at the reaction unit**



Adsorption isotherm of cell extract



CURRICULUM VITAE

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Work Experience:

12.2005 – 05.2009 Scientific coworker at the Biochemical Engineering Department at RWTH Aachen University. Research Topic: “Biocatalysis in Non-Conventional Media”.

09.2004 – 02.2005 Trainee at Bayer Technology Services – Bayer AG. Topic: “Cloning and expression of a growth factor from mouse cells. Screening by means of co-expression of the Green Fluorescent Protein (GFP)”.

Education:

10.2003 – 10.2005 Master in Biotechnology at the Technical University of Hamburg – Harburg. Master Thesis: “Analysis of the product and by-products formation during high cell density fermentations of recombinant E.coli”.

10.1998 – 07.2003 Diploma in Chemical Engineering at the National Technical University of Athens. Diploma Thesis: “Isolation and Characterization of an esterase of ferulic acid from the thermophilic yeast *Sporotrichum thermophile*.”.

09.1995 – 06.1998 Lyceum (Argyroupolis – Attika)