Application of magnetic separation technology for the recovery and re-use of immobilised lipase of *Candida antarctica* A-type (CALA)

Dissertation

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„ Prediction is very difficult especially about the future “

Niels Bohr

Preface

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Summary

The starting point for this project derived from the observation that in down stream processing and in biocatalytic, enzymatic conversions there have often been reported problems when it comes to the realisation of such processes on a technical scale. Enzymatic biocatalysis is often performed in particulates containing feed stocks, viscous or two phase systems with free enzymes. In general, those free enzymes can not be recovered and recycled after the reaction is finished. This provokes a relatively high production cost for biocatalytic processes per kg of the manufactured product. Thus, enzymatic based production is only cost-worthy for high priced products. Environmentaly friendly and mild biocatalytic reactions are not able to compete with well-established and well-equipped chemical processes. Furthermore, the use of enzymes entrapped in materials containing gel very often leads to diffusion limitations and leads to bio fouling. The biocatalytic result of those reactions is not very promising and has the potential for optimisation.

Thus, the study presented in this thesis shows the proof of principle for a semi-continuous multicycle re-use of immobilised CALA in a model viscous and 2-phase-system from oil and water. Multiple recovery and re-use of immobilised enzymes on magnetic micro particles (1 µm) have been performed in a mini pilot scale using HGMS for the first time. The applicability of HGMS for the recovery and re-use of immobilised CALA was shown in water based, viscous, and 2-phase-systems consisting of a model grease emulsion (tributyrin/buffer). Different areas of investigation have been necessary for the subsequent development of this newly researched system for the multiple recovery and re-use of immobilised enzymes in a magnetic filter using HGMS in a mini pilot scale.

The first step in this thesis was to develop a basic assay (chapter 6) for the direct determination of the activity of immobilised lipases on magnetic particles. This work increases the possibility of measuring the activity of magnetic support immobilised lipases directly in a spectrophotometric assay in a very short and efficient time period of less then 5 min. It was thus examined, for the first time, how the common *p*-nitrophenyl palmitate based spectrophotometric measurement of free lipases could be adapted to provide a reliable assay suitable for screening lipase activity after

enzyme immobilisation on different functionalised magnetic particles. Three different types of lipase (*Candida antarctica* lipase A and B and *Thermocatenulatus lanuginosus* lipase) were immobilised on two different types of magnetic particles and the activity of the resultant immobilised enzyme preparations was measured directly in the reaction solution by using a modified *p*-nitrophenol ester assay. Removal of the solid particles was not necessary prior to spectrophotometric measurement, thus allowing reliable kinetic measurements to be made in a rapid manner. The optimal concentration of insoluble support in the spectrophotometer cuvette was found to be between 0.01 and 0.2 mg ml^{-1}. In all cases, the assay could be used to determine the bead related specific enzyme activity and was thus employed for screening experiments (i.e. lipase and magnetic particle type, as well as activation chemistry). The assay was validated by comparing with a pH-stat method using *p*-nitrophenol palmitate as the substrate and we found excellent correlation between the two methods. The utility of the spectrophotometric assay was demonstrated by applying it to identify the best combination of lipase type, activation chemistry and magnetic particle. It was found that epoxy activation of poly vinyl alcohol (PVA) coated magnetic particles prior to immobilisation of commercial *Candida antarctica* lipase A (CALA) gave the best preparation.

The next part of the thesis is subsequently based on the development of the spectrophotometric assay and deals with the explanation and optimisation studies of immobilised CALA on magnetic micro particles that obviously resulted in different bead related specific activities as shown in the first part of this thesis (Chapter 7). Thus, optimisation studies of immobilised lipase (*Candida antarctica* A-type (CALA) on one type of magnetic micro particle (1µm) with three different terminations have been investigated (M-PVA). Widely used methods for immobilisation of enzymes such as the use of epoxy, amine and carboxy terminated carriers were chosen as a basis for comparative studies on the amount of protein coupled and on the bead related specific activity of immobilised CALA. The results indicated, similar to the first part of the thesis, that epoxy activation is preferred for lipase coupling to M-PVA magnetic beads, giving higher activity (~600 U g^{-1} bead) consuming less free enzyme. Immobilised CALA on amine (~275 U g^{-1} bead) or carboxy terminated micro magnetic adsorbents (~40 U g^{-1} bead) resulted in significantly lower bead related

specific activities. Furthermore, storing stability of free and immobilised CALA were investigated at different temperatures (25 °C, 4 °C). The ultimate aim was to define the optimal immobilisation conditions. Thus, the influence of the electrostatic interactions on the amount of protein coupled was investigated. The zeta-potential and the particle size distribution of the magnetic adsorbents and of CALA were also determined and the titration curves of the enzyme (IEP CALA ~pH 4) and the particles (IEP epoxy ~pH 4, amino ~pH 7.2) were measured. To conclude the measurements, the influence of the buffer (ionic strength, pH value) on the surface potential of CALA and on the epoxy terminated adsorbents was observed and it was shown that appropriate buffer conditions enhanced the bead related specific activity of CALA for ~30 % to a value of ~900 U g^{-1}. These results demonstrate that the efficiency of binding during enzyme immobilisation depends not only on the chemical properties of enzyme and the matrix particle, but also on their surface potential. Zeta-potential quantifies the electrostatic interactions between enzyme and matrix particles, and can therefore, be used as an indicator of the binding efficiency and as a diagnostic tool in the enzyme immobilisation studies.

Another part of the thesis concludes the basic studies described in parts before and resulted in the investigation of the proof of principle, for a semi continuous multicycle re-use of immobilised CALA in a model oil-water 2-phase-system (chapter 8). For the first time, results showing the development and semi continuous multicycle re-use, of immobilised lipases (from *Candida antarctica* A-type, CALA) on magnetic micro particles (polyvinyl alcohol (PVA), 1-2 µm) with epoxy functionalisation were produced in this thesis. The immobilised CALA was used to hydrolyse a model oil-water 2-phase-system composed of a phosphate buffer with tributyrin at up to 3 litre scale. The immobilised enzyme was subsequently recovered in a magnetic filter using high-gradient magnetic separation and reapplied in repeated cycles of hydrolysis and recovery. Two different temperatures of 30 °C and 50 °C, tributyrin concentrations (0.12 g l^{-1} and 35 g l^{-1}) and reaction times were tested. In each case the reaction was followed by pH titration using NaOH, as well as by HPLC analysis. Consecutive cycles were conducted for each reaction condition and in total the immobilised CALA was subjected to 20 recovery and re-use cycles, after which ~14 % of the initial specific activity still remained.

This newly developed model system clearly demonstrates that the recovery of immobilised enzymes in a magnetic filter using HGMS and their recycling is possible in mini pilot-scale. This principle promises that it could potentially be transformed to other industrially important enzymes (e.g. proteases, lactases) or for two-enzyme one pot systems where one of the enzymes must be separated after the first reaction step. Furthermore, immobilised proteases on magnetic particles could be employed for the removal of tags of proteins of pharmaceutical relevance after their affinity purification. Different investigation parts have been necessary for those studies and will shortly be introduced in the following sections of this thesis. Another interesting field of application of immobilised lipases in combination with HGMS separation technology would be the transformation of waste greases into methyl esters for biodiesel and lubricants. CALA is especially suitable for the degradation of waste grease as it shows preferences for the degradation of unsatturated fatty acids mainly present in food waste grease. The importance of CALA for the production of biodiesel or lubricants from waste grease or waste fats has already been thoroughly researched and can be found in various research literature.

1 Aim and motivation of the thesis

The aim of the thesis was to show the proof of principle of a simple and robust procedure for the multiple recovery and re-use of immobilised enzymes from viscous media, suspensions or 2-phase-systems (oil/water) using magnetic particles and magnetic separators. Furthermore, the feasibility of the biocatalytic conversion of hard to solve substrates or substrates in suspensions or emulsions with immobilised enzymes on magnetic carriers has been investigated.

The model system for the enzymatic conversions is mainly represented by the lipase of *Candida antarctica* A-type (CALA) working in a 2-phase-system of oil and water. Micro magnetic particles (1 µm) from Chemagen (Bio-Polymer Technology AG, Baesweiler, Germany) were employed. A high gradient magnetic separator (HGMS) suitable for the needs of the biological sector was developed by the research centre of Karlsruhe and could be used in the experiments.

The project was motivated from the observation that in down stream processing and in biocatalysis, problems with enzymatic conversions can be observed when it comes to the technical realisation of such processes. In the case of enzymatic biocatalysis performed in particulates containing viscous or 2-phase-systems, the reactions are performed with free enzymes. These free enzymes can not generally be recovered and recycled after the reaction is finished. Thus, the enzyme costs for biocatalytic processes per kg of the manufactured product are relatively high and only cost worthy for high priced products. These environmentally friendly and mild biocatalytic reactions are therefore not able to compete in comparison to existing well-established and well-equiped chemical processes. Moreover, the use of immobilised enzymes on conventionally used porous particles or the use of enzymes entrapped in gel containing materials very often leads to diffusion limitations and bio fouling problems. The result of the biocatalysis in such systems is thus not very promising for the most part and leaves room for optimisation.

Keeping these problematic points in mind, it is well known that the application of magnetic separation technology in bench scale for the efficient separation of DNA-products and tagged proteins as well as for assorting of cells has already been

widely described in literature. The affinity purification of tagged proteins in mini pilot scale has only recently been described in the review by Franzreb (2006). The principle of magnetic separation is shown in figure 1.

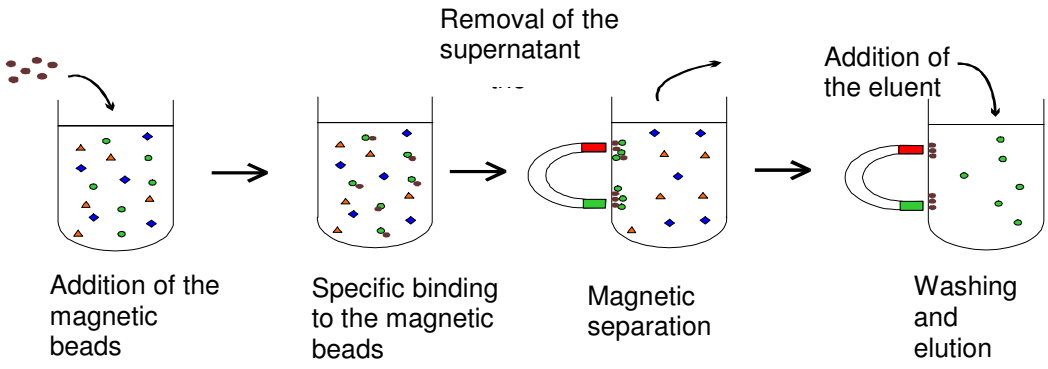

Figure 1: Principle of magnetic separation technology (in courtesy of Matthias Franzreb)

Bench scale investigations of immobilised enzymes on magnetic carriers were performed for the first time in the 1980s. It has already been shown that immobilised enzymes on non-porous magnetic particles can be used for the conversion of substrates in particulates containing media.

However, the original ideas of large-scale enzyme immobilisation and bioseparation as well as large-scale affinity purification have gained less interest and are not yet transfered to the technical scale. The main reasons for this have been the lack of cost-effective magnetic carriers with the properties of scaled-up production. Until recently there have not been designed any magnetic separators that were suitable for the needs of biological demands. Close cooperation between biologists, chemists and engineers is needed for the complex investigation of a new separation technique in the bio-sector.

Different areas of research for the recovery of lipase from *Candida antarctica* immobilised on magnetic micro particles with integrated processing and multiple reusability of enzymes by magnetic separation technology have been employed in this thesis. The main areas of research are shown in figure 2 and explained in the outine of this thesis.

2 Outline of the thesis

The studies presented here focus on the immobilisation of lipases and more specifically on the immobilisation of lipase from *Candida antarctica A-typ* (CALA), as a promising biocatalyst. Optimisation studies of immobilised CALA on non-porous micro magnetic particles (Chemagen Bio-Polymer Technology AG, Baesweiler, Germany) terminated with amino-, epoxy or carboxy groups have been undertaken and suitable assay systems for screening of immobilised lipases have been developed for aqueous and viscous media. A new basic spectrophotometric assay system for the determination of micro magnetic particle immobilised lipase activity has been developed.

These basic studies have been gathered together and concluded in the investigation of the proof of principle for a semi continuous multicycle re-use of immobilised CALA in a model oil-water 2-phase-system. The new results presented here on the multiple recovery and re-use of immobilised enzymes have been performed using HGMS technology in mini pilot scale for the first time. This system was choosen to show the applicability for HGMS and immobilised CALA not only in aqueous, but also in viscous systems consisting of a model grease emulsion (tributyrin/buffer). The importance of CALA for the production of biodiesel or lubricants from waste grease or waste fats has already been thoroughly investigated by Brenneis (2004) and Hesselbach (2003). The lipase CALA is especially suitable for the degradation of waste greases as it shows preferences for the degradation of unsaturated fatty acids mainly present in food waste greases.

This newly developed model system clearly demonstrates that the recovery of immobilised enzymes in a magnetic filter using HGMS and their recycling is possible in mini pilot-scale. This principle promises that it could potentially be transformed to other industrially important enzymes (e.g. proteases, lactases) or for two-enzyme one pot systems were one of the enzymes must be separated after the first reaction step. Furthermore, immobilised proteases on magnetic particles could be employed for the removal of tags of proteins of pharmaceutical relevance after their affinity purification. Different areas of research have been nesessary for those studies and will be

introduced in the following section. The main points important for the understanding of the thesis are shown in figure 2.

1. *Development of a suitable assay system:* An assay for the direct determination of the activity of immobilised lipases on magnetic particles had to be established. Different types of lipases were immobilised on different types of magnetic particles and the activity of the resultant immobilised enzyme was measured directly in the reaction solution by using a modified *p*-nitrophenol ester assay. Removal of the solid particles was not necessary prior to spectrometric measurement, thus allowing reliable kinetic measurements to be made rapidly and for screening of different immobilised lipases for the best and most suitable properties.

2. *Optimisation studies of lipase immobilisation:* Optimisation studies of immobilised CALA on micro magnetic particles (Chemagen Bio-Polymer Technology AG, Baesweiler, Germany) with three different terminations (amino-, epoxy or carboxy groups) have been investigated. Comparative studies on the amount of protein coupled and on the bead related specific activity of immobilised CALA were done and it was investigated that epoxy activation is prefered for lipase coupling. The zeta-potential and the particle size distribution of the magnetic adsorbents and of CALA were measured in cooperation with G. Metreveli (Water Chemistry, University of Karlsruhe) to investigate the influence of the electrostatic interactions on the amount of protein coupled. Furthermore optimal immobilisation conditions were defined and the storing stability of free and immobilised CALA was investigated at different pH values and temperatures.

3. *Multiple recovery and re-use of CALA immobilised on magnetic carriers:* The developement and semi-continous multicycle re-use of immobilised lipases on magnetic micro particles was investigated. The immobilised CALA was used to degrade a model oil-water 2-phase-system, then recovered in a magnetic filter using HGMS and applied in a new cycle (up to 20 times). The reaction of the immobilised CALA was followed by pH titration with NaOH, as well as by HPLC.

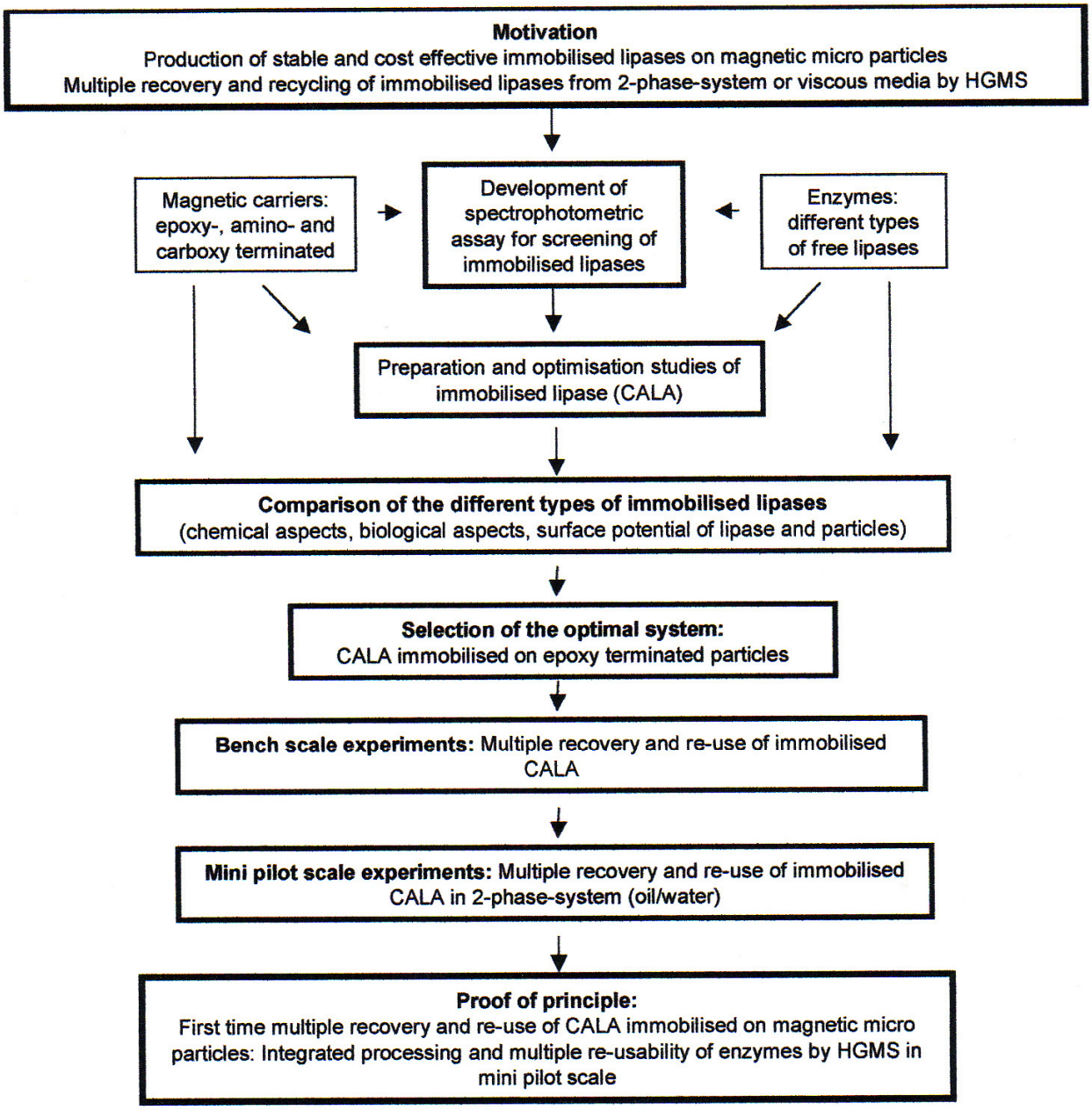

Figure 2: Main steps of research in this Ph.D. thesis

3 Introduction to the field of research

3.1 Use of enzymes yesterday and today – a brief overview

The use of biocatalysis for industrial synthetic chemistry is on the verge of significant growth (Schmid *et al.,* 2001). Today, enzymes are commonly used in many industrial applications and the demand for more stable, highly active and specific enzymes is growing rapidly. It was estimated that in 1995, the world sale of industrial enzymes would be >1.0 billion dollars, while the world market for industrial enzymes was expected to be in the range of between 1.7 and 2.0 billion US dollars by the year 2005 (Godfrey and West, 1996). Nowadays, about 60 % of the total world supply of industrial enzymes is produced in Europe, and the remaining 40 % are produced by the USA and Japan (www.tkk.fi/Units/BioprocessEngineering). About 75 % of the industrial enzymes are hydrolases with carbohydrolases representing the second largest group. Expressed in percentage, about 37 % of the industrially produced enzymes are used in detergents, 12 % for textiles, 11 % for starch industry, 8 % for baking and about 6 % for animal feed (Bhat, 2000).

Enzymes can be called the “catalytic machinery” of the living systems as most of the reactions in living organisms are catalysed by protein molecules. Man has indirectly used enzymes almost since the beginning of human history. Enzymes are responsible for the biocatalytic fermentation of sugar to ethanol by yeasts - a reaction that forms the basis of beer and wine manufacturing. Furthermore enzymes oxidise ethanol to acetic acid which has been used in vinegar production for thousands of years. Similar microbial enzyme reactions of acid forming bacteria and yeasts are responsible for aroma providing activities in bread making and in preserving activities in sauerkraut preparation. The fermentative activity of microorganisms was discovered only in the 18th century and finally proved by the French scientist Louis Pasteur. The term “enzyme” derives from Greek words, which literally mean “in yeast”. This name was given due to its close association with yeast activity (Lengler *et al.,* 1999).

The German company, Röhm, was the first to prepare a commercial enzyme called “trypsin” in 1914. Trypsin was isolated from degraded proteins from animals and

used as a detergent that became a very powerful washing powder (Burnus) (www.degussa-geschichte.de). The real breakthrough of enzymes occured with the introduction of microbial proteases into washing powders. The first bacterial *Bacillus* protease was marketed in 1959; it became a big business, when Novozymes in Denmark started the production, and a major detergent manufacturer started to employ the product, around 1965 (Workshop- Industrial Enzymes for Food Production, Past, Present and Future Perspectives, Brasilia May 6th, 2002). In the food industry, enzymes were already used in 1930 in fruit juice manufacturing. Pectinases contain many different enzyme activities and were used for the clarification of fruit juice. The major usage of microbial enzymes in the food industry started in the 1960s in the starch industry. The traditional acid hydrolysis of starch was completely replaced by α-amylases and glucoamylases, which could convert starch with over 95 % yield to glucose. The starch industry became the second largest user of enzymes after the detergent industry (Bhat, 2000).

Presently, more than 2000 different enzyme activities have been detected and characterised. The sequence information of a growing number of organisms opens up the possibility of characterising all the enzymes and organisms on a genomic level. Baker's yeast has 7000 genes coding for about 3000 enzymes. The number of reported 3-dimensional enzyme structures is rapidly increasing. In the year 2000, the structure of about 1300 different proteins was known (www.tkk.fi/Units/Bioprocess-Engineering). The enzymes are classified into six major categories based on the nature of chemical reaction that they catalyse. Briefly:

1. Oxidoreductases catalyse the oxidation or reduction of their substrates
2. Transferases catalyse group transfer
3. Hydrolases catalyse bond breakage with the addition of water
4. Lyases remove groups from their substrates
5. Isomerases catalyse intramolecular rearrangements
6. Ligases catalyse the joining of two molecules at the expense of chemical energy.

4 State of the art- basic knowledge

4.1 Industrial meaning of biocataysis today

Biotransformations are reactions catalysed by either growing cells, sleeping cells, or isolated enzymes. Since the beginning of the last century biocatalysis has been used for industrial production in pharmaceutical processes in food industries. In table 1, important historical dates for the development of industrial biocatalysis are summarised (Syldatk *et al.,* 2001). The main developments were achieved by the use of immobilised enzymes in organic and not polar solvents.

Table 1: Historical development of biocatalysis in the chemical industries (in courtesy of Syldatk *et al.,* 2001)

Year	Process	Application
Since 1815	Empirical use without knowing the basic	Production of acetic (acid bacteria on wood chips)
Since 1945	Biotransformations with resting cells	Transformations of steroids for the production of hydrocortison and prednisolon by fungi
Since 1969	One-step reactions without the regeneration of cofactors	Glucose isomerase, amino acylase, penicillin acylase
Since 1979	Direct use of enzymes in apolar, organic solvents	Ester synthesis of lipases in isooctan
Since 1982	Recombinant enzymes	Penicillin acylase with E. coli
Since 1985	Multi-enzyme reactions with regeneration of cofactors	Enzyme membrane reactor, hold back of coenzymes by derivatisation (PEG-NAD)
Since 1990	Protein evolution by „Error-Prone-PCR“	Improvement of enzyme stability and stereo- and substrate specificity
Since 1995	„Tailor-made“ whole cell biocatalysis	Combination of enzymes of different origins for new reaction sequences

Generally, enzymes are remarkable catalysts that are capable of accepting a wide variety of complex molecules as substrates and are exquisitely selective, catalysing

reactions with unparalleled chiral (enantio-) and postitional (regio-) selectivities. Therefore, biocatalysts can be used in simple and complex transformations without the need for the tedious blocking and deblocking steps that are common in enantio- and regioselective organic synthesis. In addition, the higher selectivity of enzymes affords efficient reactions with few by-products, and thus enzymes present an environmentally friendly alternative to conventional chemical catalysts (Schmid *et al.,* 2001). Most of the commercial enzymatic processes today share several characteristics such as high product concentrations and productivities, lack of undesirable by-products, and the use of enzymes that do not require expensive cofactors. Enzymes and whole cells can be selected for conversions such as isomerization and hydrolysis reactions, that do not require regeneration of coenzymes. However, when cofactors are required, whole cells are favoured because they enable cofactor regenerations. It is well known that enzymes are active in organic solvents, and many work well even in pure solvents or in supercritical fluids in the absence of added water (Blinkovsky *et al.,* 1992; Klibanov 1990). The use of organic solvents has many potential advantages such as higher substrate solubility or the reversal of hydrolytic reactions and modified enzyme specificity. Apolar organic compounds, as well as water-soluble compounds, can be modified selectively and effectively with enzymes and biocatalytically active cells. This results in new enzyme activities that previously were only possible using genetic modifications or complex multistep pathways within whole cells (Schmidt *et al.,* 2001).

4.1.1 Basic concepts of the lipase chemical reaction in organic chemistry

Lipases are amongst the most important biocatalysts for carrying out novel reactions in aqueous and non-aqueous media. This is primarily due to their ability to utilise a wide spectrum of substrates, to provide high stability towards extremes of temperature, pH and organic solvents, and to show chiral regio- and enantioselectivity. In other words, for racemic alcohols, only one enantiomer is acylated, thereby leading to enantioselective transformations. Moreover, lipases are

active in organic solvents, and thus water can be replaced by other nucleophiles, such as alcohols (Figure 3).

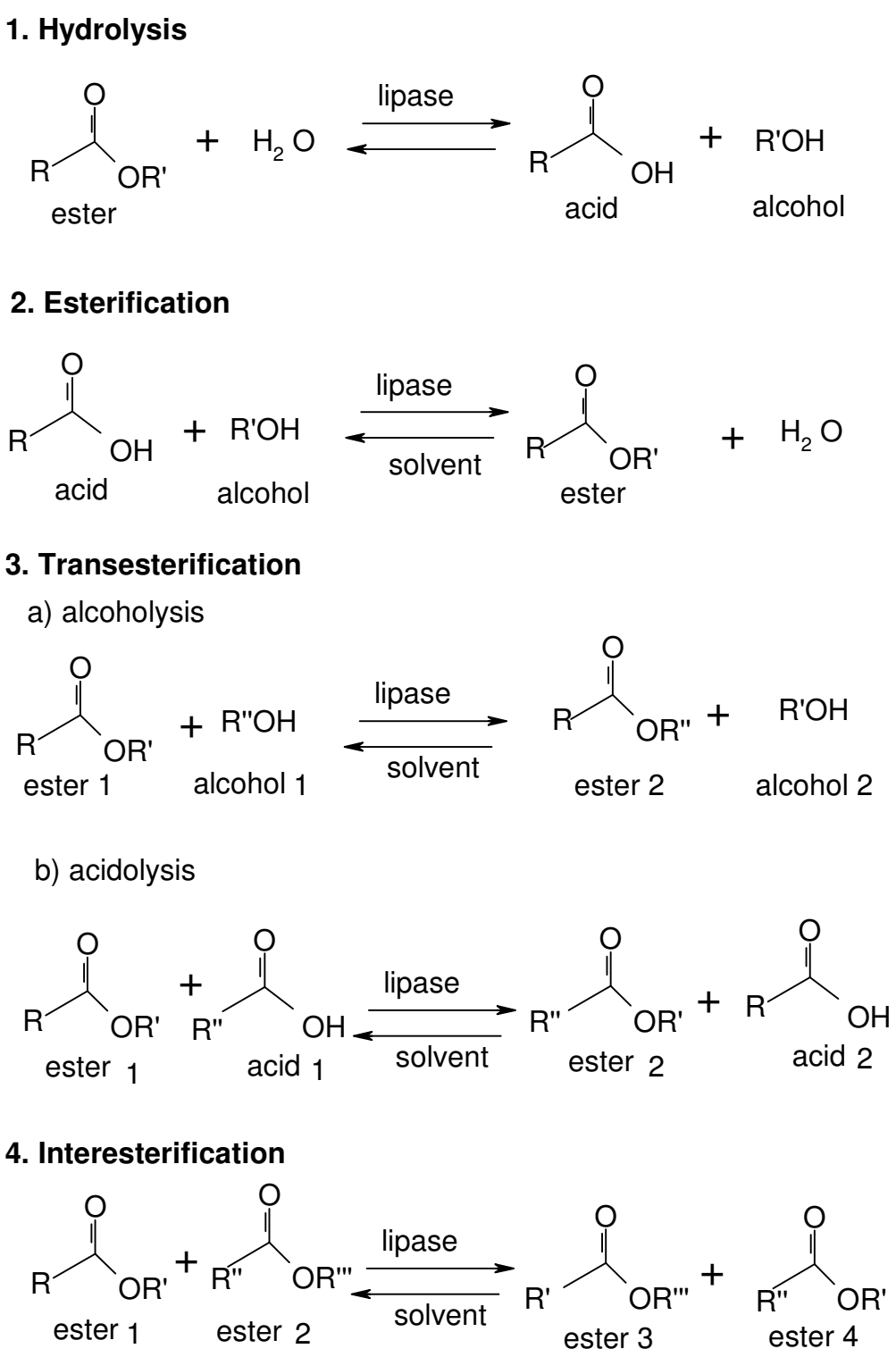

Figure 3: Basic concepts of the lipase chemical reaction in water and in non aqueous systems are hydrolysis (1), esterification (2), transesterificatioin (3), and interesterification (4)

A transesterification is the result of this reaction (Schmid *et al.,* 2001). The basic reaction of lipases is the hydrolysis of the ester bond between the fatty-acyl side chains and the lipid backbone. Lipids consist primarily of fatty-acid chains, which are linked by ester bonds to an alcohol or to a polyol backbone. In particular, microbial lipases find immense application because they can be cultivated easily, and their lipases can catalyse a wide variety of hydrolytic synthetic reactions (Gandhi, 1997). In order to understand the different research perspectives that are possible with lipases and the reactions described in this thesis, it is necessary to become acquainted with the basic concepts of lipase reactions, as shown in figures 3 and 4. In this thesis, the main reaction performed by lipases are the hydrolysis of tributyrin in buffer to free fatty acids and glycerol and the hydrolysis of para-nitrophenylpalmitate (*p*-NPP) to palmitate and para-nitrophenol (*p*-NP).

For lipase-catalysed reactions, the lid of the lipase has to be open so that the active site becomes accessible to the substrate. The investigation of the catalytic cycle of lipases has been of significant importance for their widespread use in different biotechnological applications, and this investigation will be described here based on the explanations of Schmid (2001) and Zhang (2000). Briefly, the active site of lipases is composed of three amino acid residues, namely serine, histidine, and aspartate or glutamate. The hydrolysis of an ester involves an acyl enzyme complex. The catalytic cycle starts by nucleophilic attack of the hydroxyl group of the serine side chain on the carbon atom of the ester bond. The complex is resolved by the nucleophilic attack of water, the fatty acid is liberated, and the enzyme is regenerated.

A mechanism for the lipase-catalysed hydrolysis, involving interesterification, acylation, and deacylation steps, was proposed by Zhang (2004) and is shown in figure 4. In the acylation step, a covalent acyl enzyme is formed by the nucleophilic attack on the carbonyl of the TAG by the essential Ser-OH. Deacylation of the acyl-enyzme occurs with H_2O being nucleophilic, giving the free fatty acid (FFA) and enzyme, or with diglyceride (DAG) being nucleophilic, giving the new triglyceride (TAG) and enzyme (Figure 4). Furthermore, the lipase catalysed interesterification involves the presence of water during the reaction and results in the formation of new TAG products, in addition to DAG and FFA by-products, in the system. A simplified form, in which E stands for the enzyme, is used instead of a detailed molecular

structure to describe the process of lipase catalysed interesterification between triglycerides based on the formation of an acyl enzyme complex (Zhang, 2004).

$TAG_1 + E \leftrightarrow TAG_1 \cdot E \leftrightarrow DAG_1 + FFA_1 \cdot E$ 1)
$TAG_2 + E \leftrightarrow TAG_2 \cdot E \leftrightarrow DAG_2 + FFA_2 \cdot E$ (2)

$DAG_1 + FFA_2 \cdot E \leftrightarrow TAG_3 \cdot E \leftrightarrow TAG_3 + E$ (3)
$DAG_2 + FFA_1 \cdot E \leftrightarrow TAG_4 \cdot E \leftrightarrow TAG_4 + E$ (4)

$TAG_3 \cdot E \leftrightarrow \cdots\cdots\cdots$ (5)
$TAG_4 \cdot E \leftrightarrow \cdots\cdots\cdots$ (6)

$FFA_1 \cdot E + H_2O \leftrightarrow FFA_1 + E$ (7)
$FFA_2 \cdot E + H_2O \leftrightarrow FFA_1 + E$ (8)

The reaction will continue until an equilibrium is reached involving production of new intermediates and formation of new TAG products (Eq. 1 to 6). When the water content increases in the system, the FFA content will increase, too (Eq. 7 to 8), whereas $FFA_1 \cdot E$ or $FFA_2 \cdot E$ decreases. In consequence, the content of DAG will also increase (Eq. 3 to 4). The total yield of interesterified new TAG products will decrease. The free water is the main reason for raising the FFA in the system. Zhang (2000) predicted that one mole of water in the reaction system generates one mole of FFA. Thus, the water content in the system should be controled in order to reduce the contents of FFA and DAG in the final product.

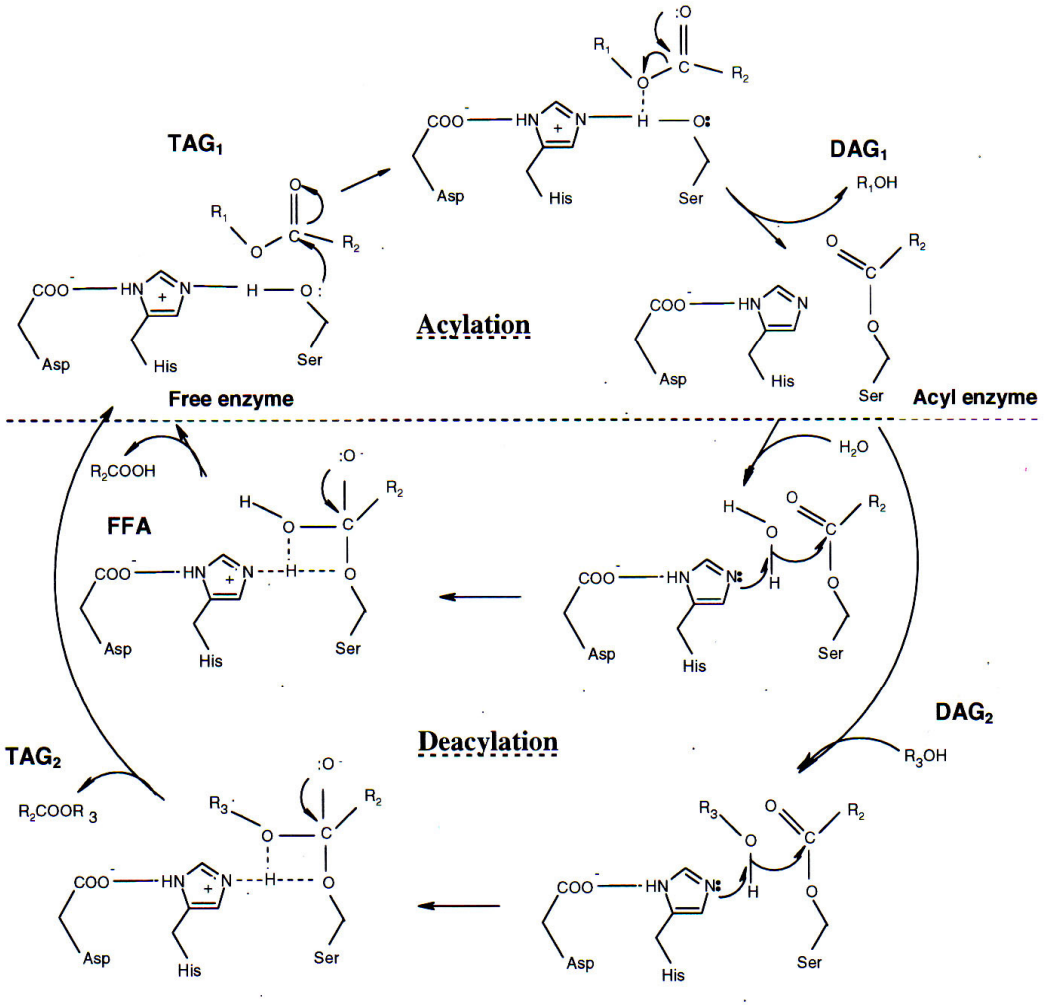

Figure 4: Proposed pathway for lipases catalysed hydrolysis, interesterification, acylation and deacylation steps (in cortesy of Zhang, 2004).

4.1.2 Lipase of Candida antarctica A-type (CALA) - special features of the enzyme

In the following, some details about *Candida antarctica* A-type (CALA) will be reported, as it was the main enzyme used for the studies. The introduction to CALA is based mainly on the very informative and excellent review of de Maria (2005) unless otherwise noted. Lipases produced by different strains of genus *Candida sp.* are among the most widely used enzymes for biocatalytical purposes. In particular, for *Candida antarctica* lipase B (CALB), an enormous number of different applications have been reported (Kirk *et al.,* 2002; Nielsen *et al.,* 1999). Originally, CALA was isolated from the lake Vanda, in Antarctica, in the late 1980s; the yeast was also isolated from natural Japanese samples (Nielsen *et al.,* 1999; www.unep.org/dewa/antarctica/PDF/bioprospecting_final.pdf). The production of

Candida antarctica lipases was optimised due to the many published applications of lipases and to the strong interest shown in these biocatalysts. Two isoenzymes (called lipase A and B) were characterised (Michiyo, 1988; Heldt-Hansen *et al.,* 1989), purified (Patkar *et al.,* 1993), and cloned and overexpressed with *Aspergillus oryzae* as host organism (Hoegh *et al.,* 1995). Much attention has been paid to the utilisation of CALB, especially in asymmetric synthesis (Kirk *et al.,* 2002; Nielsen *et al.,* 1999). CALA attracted much less interest, despite its unique properties, but during the last few years CALA has found many remarkable applications, as demonstrated by the increasing number of publications.

CALA is a calcium dependent enzyme that has a molecular weight of 45 kDa, a pH optimum of approximately 7, and an isoelectric point (*pI*) of 7.5 (Kirk *et al.,* 2002). CALA shows higher interfacial stability activation than CALB, but less than *Humicola lanuginosa* (Kirk *et al.,* 2002; Martinelle *et al.,* 1995). The N-terminal sequence of both lipases A and B is known, and there is no homology between them. In the case of CALA, there is also no similarity with other lipases (Hoegh *et al.,* 1995). In contrast to CALB, the tridimensional structure of CALA still remains unknown and the work on mutants scarce while the crystallographic structure of CALB is available, and some research on the development of new mutants has been done (Lutz, 2004). However, a new CALA with four-fold higher specific activity was developed by changing Phe 135 and Phe 139 by two Trp residues (Svendsen *et al.,* 1994). Furthermore, the total amino acid sequence of CALA and CALB has been sequenced and was published; it is shown in table 2 (Patkar *et al.,* 1993).

Table 2: Total amino acid composition of *Candida antarctica* lipases of A and B components (Patkar *et al.,* 1993)

Amino acids	A component	B component
Asp	37	27
Thr	29	26
Ser	28	30
Glu	37	22
Pro	34	29
Gly	32	25
Ala	49	35
Val	28	22
Met	2	4
Ile	25	10
Leu	31	31
Tyr	17	8
Phe	20	9
Lys	18	9
His	6	1
Trp	4	4
Arg	9	8
Cys	4	5

CALA, is considered to be the most thermostable lipase known, being able to work efficiently at > 90 °C (Kirk *et al.,* 2002; Michiyo 1988; Heldt-Hansen 1989; Zamost *et al.,* 1991). It seems quite strange that a microorganism which is able to grow under the cold conditions of Antarctica can produce such thermostable proteins. In fact, most of the hydrolases presently known are not active at temperatures higher than ca. 45 °C. Recently, some applications were reviewed (Nielsen *et al.,* 1999), including several patents that claim possibilities like hydrolysis of triglycerides in the pulp industry (Fujita *et al.,* 1992) or the wax lubricants field (Lund *et al.,* 1997) at high temperatures. Furthermore, a proper immobilisation of CALA leads to an even higher

thermostability (Heldt-Hansen 1989, Zamost *et al.,* 1991). The hydrolysis of triglycerides, and their chemical handling to enhance the proportion of certain fatty acids, represents an important application within the lipases field (Schmid *et al.,* 1998). CALA has been reported as a non-selective biocatalyst (Heldt-Hansen, 1989) as it shows an sn-2 preference, but this does not enable selective synthesis of 1,3-diglycerides or 2-monoglycerides (Kirk *et al.,* 2002). However, in general, most of the other lipases known for this application have an sn-1,3 preference. Within the lipases field, usually *cis*-selectivities towards fatty acid substrates are found. In the cases of *Candida rugosa* and *Geotrichum candidum*, the existence of a tunnel in the substrate recognition site (acyl part) was reported (Grochulski *et al.,* 1994). Such a tunnel is not straight but has an L shape which made it quite useful for the acceptance of *cis*-fatty acids. In contrast, CALA has an outstanding selectivity towards *trans*-fatty acids when compared with the corresponding *cis*-derivatives.

Moreover, CALA is considered to be an excellent biocatalyst for the asymmetric synthesis of amino acids/esters, due to its chemoselectivity towards amine groups (de Maria *et al.,* 2005). This capability makes CALA a useful catalyst in the production of enantiopure amino acids and related molecules, as has been widely discussed in the last few years (Solymar *et al*., 2002; Gyarmati *et al.,* 2003). Furthermore, CALA is capable of accepting highly sterically hindered alcohols (Bosley *et al.,* 1995), and recently, it has been discovered that such a capability is due to a special aminoacidic motif within the oxyanion binding (GGGV sequence, G: Glycine and X: any amine acid) (Henke *et al.,* 2003). This is not very common among hydrolases, and therefore, it may offer a promising research field, since such bulky structures are a useful group of building blocks (de Maria *et al.,* 2005).

4.2 Immobilised enzymes: Economical aspects and immobilisation techniques

The generally high costs of biocatalysts make their repeated or continuous use desirable in most cases. This can be explained because the re-use and the recovery of native enzymes from the reaction mixture, possibly consisting of a suspension of enzymes or other biocatalysts (cells, organelles), is difficult and uneconomical. On the other hand, there are also processes based on homogeneously suspended cells

or enzymes that are sufficiently inexpensive to permit single use without recovery or re-use (Schmid *et al.,* 2001).

The major opportunity, however, and the main target for immobilised biocatalysts could be the production of fine and speciality chemicals that have relatively low-volume and high-cost. Enzymes seem to be attractive catalysts in those cases as they show specificity of action and the ability to function under mild conditions (Klibanov *et al.,* 1983). Immobilised catalysts have proved particularly useful for the production of L-amino acids which are widely used as food additives, as animal feed, and in medicines. Normally, chemical syntheses are simpler, faster and cheaper than fermentations but almost always result in racemic mixtures of amino acids (Klibanov *et al.,* 1983) that are a big disadvantage in many cases (pharmaceuticals etc.). Klibanov (1983) stated that as long as conventional feedstocks such as oil, natural gas, and coal are used, biotechnology in general, and immobilised biocatalysts in particular, are unlikely to have a great impact on the production of bulk chemicals. The conclusion is based on the fact that 60 % to 80 % of the costs of bulk chemicals is due to the cost of the raw materials. A change in the process technology would thus have a rather small effect on the economics. Furthermore, there are excellent chemical catalysts for the synthesis of bulk chemicals, and the opportunities here appear to be unlimited. Additionally, enormous capital investments have been made in the existing chemical plants and most of them currently function well bellow capacity. Thus, the chances of sizeable investments in novel and competing technologies are low. In 1983 the current opinion was that only when alternative feedstocks such as wastes become a major factor can one expect biotechnology to play an important role. At that time, assuming that wastes would become more important, it was predicted that this would not happen before 30 to 50 years later than 1983 (Klibanov *et al.,* 1983). Now, 20 years later, the present work focuses on the development of a semi continuous and multicycle re-use of immobilised lipases (CALA) on micro magnetic particles (M-PVA) for the degradation of a model oil-water 2-phase-system. The model system is used for the purpose of investigations as a substitute for a real waste grease/water mixture in future works (greases from restaurants, slaughter houses or the metal industry).

Briefly, some of the most popular examples of immobilised biocatalysts that are used for industrial processes are mentioned in table 3. Those processes will not be further

discussed here, and a general introduction to possible immobilisation techniques for enzymes will be given. Excellent reviews and text books have been written for further information about the processes (Buchholz *et al.,* 2005; Klibanov *et al.,* 1983; Hartmeier *et al*., 1985).

Table 3: Examples for immobilised biocatalysts used in industrial processes (adapted from Hartmeier *et al.,* 1985).

Biocatalysts	Method of immobilisation	Application
L-amino-acylase	Ionic binding to anion exchangers	L-Amino acids from synthetically made mixtures of D- and L- amino acids
Glucose isomerase	Co-crosslinking with gelatin or immobilisation of dead cells	Fructose containing syrups (HFCS) from glucose
Penicillin acylase	Carrier binding or immobilisation of dead cells	6-Amino penicillanic acid for the production of semisynthetic penicillins
L-aspartase	Entrapment of dead *E. coli* cells	L-Aspartic acid from fumaric acid
Lactase	Entrapment into cellulose acetate fibres	Lactose hydrolysis in milk
Transglycosidases	Entrapment of cells into alginates	Transglucosidation of saccharose to isomaltulose

In general, carrier-bound, carrier-free (e.g. cross-linked enzyme aggregates, cross-linked enzyme crystals, etc.), and immobilised enzymes (Cao *et al.,* 2003) can be distinguished. The choice of the matrix used for the attachment of the biocatalyst is determined by several requirements such as specific properties (morphological, chemical, mechanical), economical considerations (reliable source at a reasonable price), and separation issues. The immobilisation of isolated enzymes, by definition, is the conversion of enzymes from a water-insoluble, mobile state to a water-insoluble, immobilised state. More than 100 immobilisation techniques have been elaborated until now and can be divided into the following five groups (Klibanov *et al.,* 1983) that are summarised briefly here (Figure 5). (a) The first group includes the covalent attachment of enzymes to a variety of solid supports such as porous glass, ceramics, cellulose, synthetic polymers, stainless steel, charcoal, and metallic oxides. Enzymes are usually immobilised through their amino- or carboxyl groups. In

most instances, the immobilisation procedure consists of at least two steps, the activation of the support and the attachment of the enzyme. (b) The second group consists of enzymes that are adsorbed on solid supports (e.g., diethylaminoethyl cellulose (DEAE-cellulose), Sephadex). The simplicity is the attractive feature of adsorption immobilisation. Most proteins are readily adsorbed on ion-exchangers and therefore they have been widely used for enzyme immobilisation.

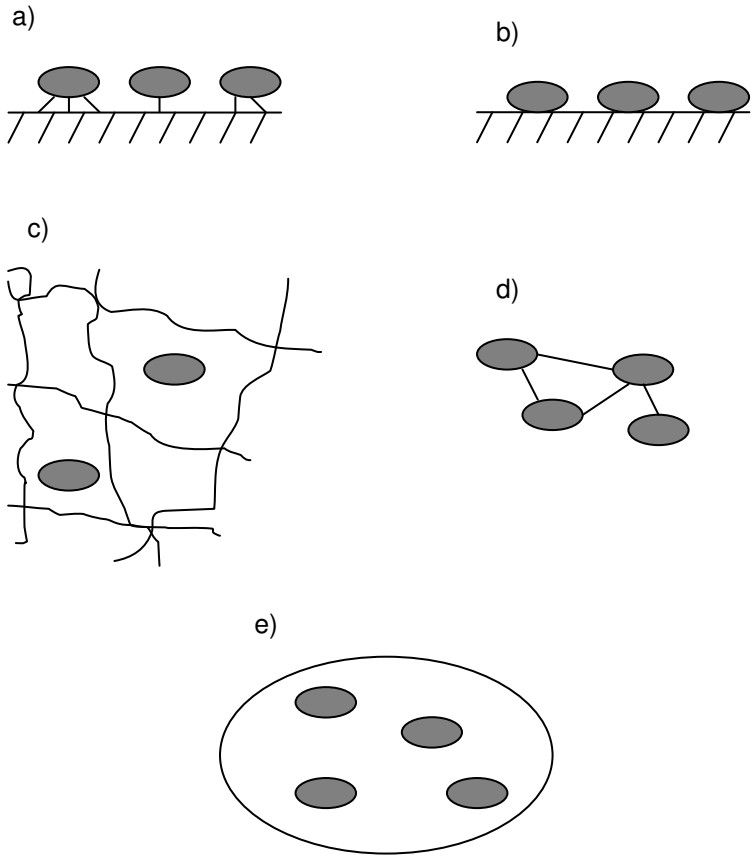

Figure 5: Methods of enzyme immobilisation: (a) covalent attachment to solid supports, (b) adsorption on solid supports, (c) entrapment in polymeric gels, (d) intermolecular cross-linking, and (e) encapsulation (in courtesy of Buchholz *et al.,* 2005).

(c) In this approach, enzymes are trapped in polymeric gels. The enzyme is added to a solution of monomers before the gel is formed. The gels employed for immobilisation of enzymes may be covalent (e.g., polyacrylamide cross-linked with N,N'-methylenebisacrylamide) or noncovalent (e.g., calcium alginate). (d) Using this method, the enzymes are cross-linked with bifunctional reagents, e.g., glutaraldehyde, that cross-link the enzyme through amino groups. Another example of reagents would be diamines (for instance, hexamethylene diamine) that cross-link

the enzyme through carboxyl groups following activation of these groups with carbodiimides. (e) This method encapsulates enzymes in various types of membranes that are impermeable for enzymes and other macromolecules but permeable for low molecular weight substrates and products. Several reviews about immobilised enzymes used in industry or food applications, and also about enzyme membrane reactors, can be found in the literature (Hartmeier *et al.,* 1985, Klibanov *et al.,* 1983*;* Christensen *et al.,* 2003; Rosevar, 1984).

Currently, enzymes are typically immobilised on large porous particles (100-200 µm) with high specific surfaces areas which allow high enzyme loadings. The particles are packed in columns or added batch wise to a reaction mixture and recovered by centrifugation. Such large porous particles suffer from diffusion limitations (Bozhinova *et al.,* 2004) for the substrates and/or the products during the course of the enzyme catalysed process, their separation will be difficult, if the reaction solution is dense or viscous, and particulates must be avoided in the feed. Moreover, fouling of the matrix pores presents a difficult task when reactions in suspensions are performed or when poorly soluble substrates or products are used. All of these problems can potentially be avoided by using non porous and smaller (1 µm or less) particles. However, the range of the separation options for such supports is very small. In this context, magnetic carrier technology offers a new, convenient, method for the separation of small (1 µm or less) particles. The application of magnetic matrices for the purpose of biotechnology and molecular biology is very promising (Dunlop *et al.,* 1984 Setchell 1985). The development of smaller, non-porous, and relatively easy-to-separate magnetic matrices thus can be favourable for several biotechnological processes.

4.2.1 Magnetic support materials for enzyme immobilisation

The manufacture of magnetic support materials will now be described, as this process provides the basic material for the lipase immobilisation performed in the present studies. The basics of the manufacturing process of the magnetic carriers is based on the excellent contributions of Pieters (1992), Franzreb (2006) and Bozhinova (2004). In the following, detailed classification of the magnetic matrices according to their synthesis and their application can be found. Mainly, there are three different methods for the production of magnetic carriers. Ferrites are available in large amounts and can be used as magnetic core materials (MO Fe_2O_3). In

general, magnetic iron oxide magnetite (FeO Fe_2O_3 or Fe_3O_4) is used as a magnetic core material and can be synthesised form 1 Fe^{2+}/2 Fe^{3+} salts in aqueous solutions – heated up to 70°C and precipitated by increasing the pH. Another option is the isolation of iron oxide from magneto-tactic algae or bacteria (Bozhinova, 2004).

Briefly, one manufacturing method is the coating of magnetic core materials by polymer adsorption, silanisation, graft polymerisation, or co-precipitation. In the first case, different natural or synthetic polymers can be electrostatically adsorbed onto the surface of charged magnetic particles and thus provide coatings for further chemical modification. Silane coupling chemicals are utilised to introduce reactive organo-functional moieties (mostly primary amino- or epoxy groups) to the magnetic crystals or particles (Hubbuch *et al.,* 2001). The best known micro-sized silane-coated magnetic carrier is BioMag (Polyscience, Inc., UK).

The polymer grafting method involves polymerization of a suitable monomer, forming a thin layer on the surface of silanised magnetic particles (Halling & Dunnill 1980, Hubbuch *et al.,* 2001). Co-precipitation of $2Fe^{3+}$ and $2Fe^{2+}$ salts in aqueous solutions under alkaline conditions in the presence of a suitable polymer (dextran, chitosan) provides an elegant method for the synthesis of magnetic core matrices (Yen, 1981).

Another possibility for the production of magnetic matrices is the encapsulation of magnetic solids within natural gels or synthetic polymers. An example of these magnetic carriers would be the M-PVA particles (Chemagen Polymer Technology AG, Baesweiler, Germany) used in this study; their manufacture is described by Müller-Schulte (2001). Briefly, the process is called water-in-oil polymerisation wherein the water phase consists of an aqueous polyvinyl alcohol solution in which suitable surfactants and magnetic ferrofluids are dissolved or dispersed. The oil-phase is a common vegetable oil. The homogenised (ultrasound treatment) water phase is suspended by mixing (speeds up to 2000 rpm) into the oil phase. This step is followed by addition of a cross-linker in order to create (within minutes) a polymer network for entrapment of the sub-micron sized magnetic material. This results in particles of a size of 1-10 µm that are non-porous and spherically shaped.

An additional method for the production of magnetic matrices is the infiltration of porous matrices with fine magnetic sub-micron particles, or aqueous mixtures of Fe^{2+} and other metal ions (e.g. Fe^{3+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}) that are capable of forming

magnetic ferrites. Polymer particles of uniform size are synthesised and then suspended in an aqueous Fe^{2+} salt solution; the Fe^{2+} permeates into the particle pores. In the particles, a part of the Fe^{2+} is oxidised and then transformed to magnetite and maghemit with the help of heat. In the end, the synthesised magnetic particles are treated with polymers to fill remaining gaps and to coat the surface. A kind of post magnetisation of particles has been shown by Mosbach & Anderson (1977); they infiltrated and trapped magnetic particles into commercially available sepharose or sephadex supports. A large number of magnetic adsorbent particles of different origin are currently commercially available; they are mostly listed in the internet (http://www-magneticmicrosphere.com). For further reading on the use of magnetic particles in different bioassays, see Haukans & Kvam (1993), or for various clinical applications, see Häfeli (1997).

4.2.2 Necessary properties of magnetic particles for the use in bioseparation

Based on decades of experience and for practical use, some necessary criteria that magnetic carriers should fulfill when employed in the bio-sector will be pointed out in the following paragraph. Many of those criteria for evaluating the performance of magnetically-responsive supports are similar to those for more conventional adsorbents, e.g., surface area, ligand density, binding capacity, a surface compatible retention of biological form and activity, absence of non-specific adsorption effects, easy cleaning, and particle size uniformity. Furthermore, iron oxides used to introduce magnetism must be protected from the environment to prevent leaching of iron ions to the surroundings (Heebøll-Nielsen, 2002). For particle design, certain unique magnetic properties have to be considered.

The aggregation tendency of the carrier due to remanent magnetism can be avoided by using superparamagnetic (see theoretical background) materials. Earlier, magnetically-susceptible supports were prepared with ferromagnetic (see below) materials (Halling & Dunnill 1979a; Halling & Dunnill 1979b), and the residual magnetisation characteristics of the ferromagnetic materials caused permanent aggregation of particles and thus significantly limited their use in biological applications. Thus, the prefered carrier material, M-PVA magnetic particles (Chemagen Polymer Technology AG, Baesweiler, Germany), is superparamagnetic and contains iron oxide. Furthermore, the magnetic susceptibility of the carriers is

another important criteria for the separation techniques, and high susceptibility will augment the effectiveness of the magnetic separation in general. However, high amounts of a magnetic material within the matrix cause higher magnetic susceptibility (see theoretical background) but increase the overall density of the carriers. Thus, the settlement by gravity becomes more rapid (Pieters *et al.,* 1992). The non-magnetic aggregation can be partially controlled by adapting the particle size or the surface chemistry or by choosing proper suspending electrolytes (Bozhinova, 2004). The superparamagnetic supports with sizes of 0.5-1 µm and magnetic susceptibility >35 A m^2kg^{-1} (ρ = 2-4 g cm^{-3}; magnetic contents of 50 % or higher) used in this study settle very slowly, but are rapidly separable by moderate magnetic fields (Franzreb *et al.,* 2006).

Another important criterion for the design of magnetic adsorbents is to consider that porous supports can become plugged with biological foulants, and these contaminating substrates are difficult to remove (Munro *et al.,* 1977, Halling & Dunnill 1980). In contrast, non-porous supports are less prone to fouling and easier to clean and, therefore, potentially more useful in purification from fouling feed streams (O'Brien *et al.,* 1996). However, in order to obtain a comparable surface area to typical macro-porous particles of 100 µm size, the dimensions for a non-porous support have to be of the order of 0.1-1µm (O'Brien *et al.,* 1996). A very convenient, and also unique, method for recovery of such small particles in the presence of biological debris of similar size is magnetic separation (O'Brien *et al.,* 1996). In addition, the power and efficiency of magnetic separation is especially useful at large-scale (Setchell, 1985). Thus, in the studies here, non-porous and 1 µm sized particles were used (Chemagen Polymer Technology AG, Baesweiler, Germany) for lipase immobilisation, and multiple re-use and recovery was used with the help of high gradient magnetic separation technology (HGMS).

4.3 Potential uses of lipases (hydrolases) for biocatalytic processes

A short overview of the field of possible uses of lipases is given in table 4. Lipases are used extensively in the dairy industry for the hydrolysis of milk fat. Current applications include flavour enhancement of cheese, acceleration of cheese ripening, manufacture of cheese-like products, and lipolysis of butter fat and cream (Gerhartz

1990, Arnold *et al.,* 1975; Colburn *et al.,* 1969; Kosikowski *et al.,* 1976 and Falch, 1991). The use of enzymes in washing powders still remains one of the biggest markets for industrial enzymes (Arbige *et al.,* 1989). The world-wide trend towards lower laundering temperatures has led to much higher demand for household detergent formulations. Intensive screening programmes, in combination with genetic manipulation, have resulted in the introduction of several preparations, e.g., Novo Nordisk‘s Lipolase (*Humicola* lipase) expressed in *Aspergillus oryzae* (Saxena *et al.,* 1999).

The scope of application of lipases in the oleochemical industry is enormous, as they save energy and minimise thermal degradation during hydrolysis, glycerolysis and alcoholysis (Arbige *et al.,* 1989). An example would be the production of ricinoleic acid as a valuable starting material for a variety of technical products. It cannot be produced from castor oil by conventional steam splitting process owing to side reactions, such as dehydration, interesterification, and so on. All these unwanted side reactions can potentially be avoided by using lipase, such as that from castor seed for the hydrolysis of castor oil (Mukherjee, 1994). Furthermore, the ability of lipases to hydrolyse lipids and to thereby obtain fatty acids and glycerol has enormous industrial applications. For instance, fatty acids are used in soap production (Hoq *et al.,* 1985). Lipases used for this purpose include those from *Candida rugosa* (Anonymos, 1981), castor bean (process already on commercial scale) (Stirton 1964), and *Pseudomonas fluorescence*. Miyoshi Oil and Fat Co., Japan, reported commercial use of lipase in the production of soap (Saxena *et al.,* 1999).

Table 4: Examples for the industrial applications of microbial lipases (Saxena *et al.,* 1999)

Industry	Effect	Product
Bakery	Flavour improvement and shelf life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Chemicals	Enantioselectivity	Chiral building blocks and chemicals
Cleaning	Synthesis, hydrolysis	Chemicals, removal of cleaning agents like surfactants
Cosmetics	Synthesis	Emulsifiers, moisturising agents
Diary	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Flavour agents, cheese, butter
Food dressing	Quality improvement	Mayonnaise dressings and whippings
Health food	Transesterification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavour development and fat removal	Meat and fish products
Pharmaceuticals	Transesterification, hydrolysis	Speciality lipids, digestive aids

In addition, polyglycerol and carbohydrate fatty acid esters are widely used as industrial detergents and as emulsifiers in a great variety of food formulations (low-fat spreads, sauces, ice-creams, mayonnaises). Lipase from *A. terreus* e.g., synthesises a biosurfactant by transesterification of natural oils with sugar alcohols (Yadav *et al.,* 1998). Another example is the use of lipases for the synthesis of a whole range of amphoteric bio-degradable surfactants, namely amino acid-based esters and amides (Saxena *et al.,* 1999; Hills *et al.,* 1990). Unichem International has launched the production of isopropyl myristate isopropyl palmitate, and 2-ethylhexyl palmitate (Saxena *et al.,* 1999) for use as emollient in personal care products like skin and sun-tan creams, and bath oils.

Moreover, lipases are also used in pharmaceuticals and agrochemicals. The utility of lipases in the preparation of chiral synthons is well recognised and documented. An example would be the commercial application of lipases in the resolution of racemic

mixtures in the hydrolysis of epoxyester alcohols (Saxena *et al.,* 1999; Hills *et al.,* 1990). The reaction products, R-glycidyl esters and R-glycidol are readily converted to R- and (S)-glycidyltosylates which are attractive intermediates for the preparation of optically active beta-blockers and a wide range of other products. A similar technology has been commercialised to products of the optically pure cardiovascular drug Ditiazem (Kloosterman *et al.,* 1988). Generically, the stereoselectivity of lipases is useful for the synthesis of optically active polymers (Margolin *et al.,* 1987). A number of reviews on the application of lipases has been published and can be advised for further reading (Christensen *et al.,* 2003; Gandhi 1997; Saxena *et al.,* 1999).

4.3.1 Industrial biocatalytic processes realised using immobilised lipases

The following short summary about immobilised lipases used in industrial processes is based on the reviews of Schmid (2001) and Syldatk (2001) about recently developed biocatalytic systems in chemical industries. As mentioned above, enzymes do have excellent advantages compared to conventional chemical processes, e.g., mild reaction conditions (pH, temperature, pressure), high specificity (and thus only low amounts of biocatalysts are needed compared to the amount of substrate), and the possiblity to produce optical pure products. Inspite of these advantages, there is still a comparatively low number of enzymes used for industrial processes in chemical industries. The most interesting of those are mentioned in table 5.

It is well known that lipases are active in organic solvents where water can be replaced by other nucleophiles such as alcohols. The result of this reaction is a transesterification. For racemic alcohols only one enantiomer may be acylated, thereby leading to enantioselective transformations (suitable acyl donors are vinyl esters, anhydrids, or diketene). The reaction is irreversible, and the separation of the remaining alcohol and the newly formed ester is simple. This principle is now used in many reactions to produce enantiomerically pure alcohols. BASF has extended its chiral synthesis capabilities to the enzymatic resolution of racemic alcohols, and amines might also be used as nucleophiles (Balkenhohl *et al.,* 1997). Excellent yields and selectivity, together with minimal amounts of enzyme, characterise this process which has been used by BASF since 1993. The products R-amide and S-amide can

be recovered and separated by distillation, and they have high chemical and optical purities (table 5). The process mentioned is applicable to a broad range of amines, which are of considerable interest as chiral building blocks or as auxiliaries for the syntheses of bioactive ingredients (Schmid *et al.,* 2001). Another important example of lipase application in industries is the use of lipases in the transesterification and in fat industries. Generally, the commercial value of fats depends on the fatty acid composition within their structure. A typical example of a high-value asymmetric triglyceride mixture is cocoa butter. The potential of 1,3-regiospecific lipases for the manufacture of cocoa-butter substitutes was clearly recognized by Unilever and Fuji Oil (King, 1990, Saxena *et al.,* 1999). Comprehensive reviews on this technology, including the analysis of the product composition, are available (Macrae, 1985).

Table 5: Important lipase immobilised-based industrial processes in chemical companies (in courtesy of Schmid *et al*., 2001 and Syldatk *et al.,* 2001)

Immobilised enzyme and Scale tons a^{-1})	Product	Type of reactor	Company
Lipase, several thousand t a^{-1}	Enantiopure alcohols	Immobilised bed reactor	BASF
Lipase, several hundred t a^{-1}	Enantiopure amines	Immobilised bed reactor	BASF
Lipase	(R)-glycidylbutyrate	Stirred tank reactor	DSM
Lipase	(S)-methoxyisopropyl-amine	Immobilised bed reactor	BASF
Lipase (*Mucor mihei*)	Cocoa butter	Immobilised bed reactor, stirred tank reactor	Fuji Oil, Unilever

5 Theoretical background

5.1 Basis of magnetism: Different types of magnetism

Magnetic theory is described in much more detail in physics text books, e.g., Lindner (1993) or Gertsen (1997), and the materials and summaries below are based on these two references unless noted otherwise. Magnetic fields are created by moving charges. A current passing through a conductor will thus result in a magnetic field surrounding the conductor. Furthermore, the created fields themselves have an effect on moving charges, and the magnetic forces between two (infinitely) long parallel conductors, each caring a current, is used for defining the unit of current, which is the ampere (A). 1 A is the current which would produce a force between the conductors of 2 x 10 $^{-7}$ N per meter when placed in vacuum. At the beginning of the 19th century, Ampère described the force, *F,* between two current-carrying conductors placed at a distance *r* from each other by a correlation between the respective currents in the conductors, I_1 and I_2, their length, *l,* and a factor *k* (Eqn. 1-1). The factor *k* accounts for the magnetic permeability, μ, of the surrounding space.

$$F = k\,\frac{I_1\ I_2\ l}{r}; \qquad k = \frac{\mu}{2\,\pi} \tag{1-1}$$

The above equation can be simplified by introducing the term magnetic induction (magnetic flux density), *B*, which describes the effect the first conductor (source) has on the second at a given distance *r* (Eqn. 1-2). The magnetic induction has the S.I. unit tesla *(T)* [$1\ T = 1N\,s\,C^{-1}\,m^{-1}$].

$$B = \frac{\mu\ I_1}{2\,\pi\ r} \tag{1-2}$$

The magnetic flux density, B, and the magnetic field strength, H (explained below), are both units that can be used as a measure for the strength of a magnetic field. To obtain a material independent measure, the magnetic induction, B, can be divided by the magnetic permeability, μ, and this leads to the definition of the magnetic field strength H (Eqn. 1-3). The definition of H given here refers to a straight wire and has the unit A m^{-1}. The magnetic permeability, μ, means the permeability of a certain

material towards the formation of magnetic field lines, e.g., iron shows a relatively low resistance to magnetic field lines.

$$H = \frac{I}{2\pi r} \tag{1-3}$$

The magnetic induction, *B*, in a vacuum can now be described by the product of the magnetic permeability of a vacuum, μ_o, and the applied magnetic field strength *H* (Eqn. 1-4a).

$$B = \mu_0 H \tag{1-4a}$$

When a material different form vacuum is introduced between the conductors, the magnetisation, *M*, of this material has to be accounted for separately, and thus the magnetic induction, *B*, can also be expressed as shown in eqn. (1-4b). The magnetisation, *M,* can be written as the product between its magnetic susceptibility*,* χ*,* and the applied magnetic field *H* (Eqn. 1-5).

$$B = \mu_0 (H + M) \tag{1-4b}$$

$$M = \chi H \tag{1-5}$$

The magnetisation, *M,* of a material is defined as the volumetric average magnetic dipole moment and has the units A m^{-1}. In other words, it is the alignment of the individual unpaired electrons of a material when it is placed in an applied magnetic field . And the magnetic susceptibility, χ*,* can be explained as the susceptibility to such an alignment (Hubbuch, 2001). Magnetic susceptibilities, χ*,* are analysed by plotting *M* or *B* as a function of *H* (Eqn. 1-4b).

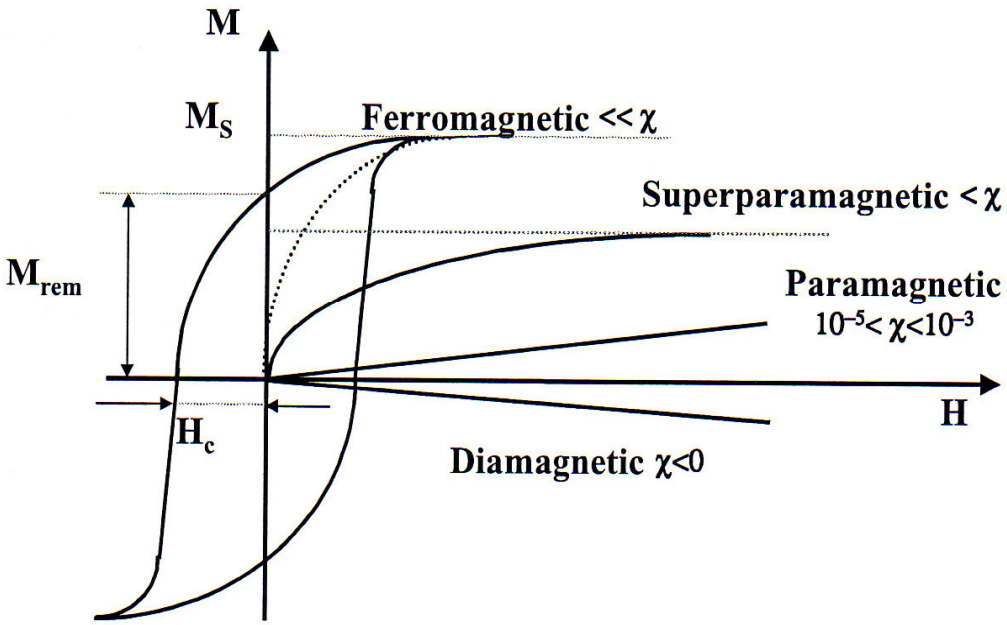

Figure 6: Magnetisation curves for dia-, para-, superpara- and ferro- magnetic materials. Magnetic materials are classified due to their magnetic susceptibility. (H) magnetic field strength; (χ) magnetic susceptibility; (M) magnetisation; (M_s) magnetic saturation; (M_{rem}) remanent magnetisation; (H_c) magnetic coercivity.

According to their magnetic susceptibility, χ, (or the localisation of the electrons in the orbitals around the atomic cores and the structure of the atoms in the substance), materials can be divided into three general groups: ferromagnetic, paramagnetic and diamagnetic. The characteristic behaviour of these groups is illustrated in figure 6, and the shape of the magnetisation curves can be seen. Ferromagnetic materials are characterised by a hysteresis behaviour when they were magnetised, and they exhibit a high magnetisation due to a high susceptibility. Once magnetised, however, these materials keep a remanent magnetisation in the absence of a magnetic field. To make the magnetisation disappear completely, a magnetic field of opposite direction to the original one is required. The magnetic field strength of this field is know as the coercivity, Hc, [A m^{-1}]. Only a few substances are ferromagnetic, and amongst these iron is probably the most obvious. Paramagnetic substances possess a much smaller magnetic susceptibility than ferromagnetic materials and loose their magnetisation when the field is reduced to zero. Diamagnetic materials have a small

negative magnetic susceptibility which leads to a repulsion of these materials in cases where the others are attracted. Examples of diamagnetic substances would be gold (Au) and Al_2O_3, and an example of paramagnetic substances would be $Mn_2P_2O_7$ and α-Fe_2O_3 (haematite) (Takayasu *et al.,* 1983). Superparamagnetic materials exhibit a behaviour that is intermediate between ferromagnetic and paramagnetic materials, as they posses a high magnetic susceptibility but no remanent magnetisation at zero field (Hubbuch, 2002b). Thus, superparamagnetic particles will respond to magnetic forces but will not remain permanently aggregated after removal of the external field. These characteristics, combined with the easy magnetisation, make superparamagnetic particles an ideal core material for supports designed for separation of biological materials intended for repeated use. A range of iron oxide materials of appropriately sized crystals shows these properties, and the limiting crystal size for the transition between ferro- and superparamagnetism of approximately 30 nm has been investigated (Hatch & Stelter, 2001). The supports used in this study for immobilisation of lipases are defined as superparamagnetic materials and were obtained from Chemagen Biopolymer-Technologie AG (Germany).

5.2 Development of High Gradient Magnetic Separation (HGMS)

The introduction to the HGMS system for bioprocesses, used in the studies here for the recovery and multiple re-use of immobilised lipases, is based mainly on the excellent review of Franzreb (2006) and on the work of Hubbuch (2002b) and Heebøll-Nielsen (2002) unless otherwise noted. Pure magnetic iron oxides such as magnetite were used for the adsorption and removal of dissolved and colloidal biological substances in waste water treatment as early as in the 1940s (Pieters *et al.,* 1991). The described technique is called high gradient magnetic separation technology (HGMS) and presently presents one of the most powerful magnetic separation methods available. In the 1970s, about 30 years later, the application of functionalised magnetic adsorbent particles for the selective capture of valuable biomolecules was described by Dunnill & Malcom Lilly at the University College London (UCL). At that time, micron-sized magnetic particles as carriers for the immobilisation of enzymes and as bioaffinity adsorbents were investigated. Bioaffinity adsorbents were used for the isolation of biomolecules directly from crude feedstocks

(Dunnhill & Lilly 1974; Halling & Dunnhill 1980). At small scale this approach became highly successful during the last two decades and numerous comprehensive reviews dealing with magnetic separation of biological molecules (Hirschbein *et al.,* 1982; Dunlop *et al.,* 1984), magnetic support technology (Pieters *et al.,* 1992), and magnetic cell separation (Owen, 1983; Lea *et al.,* 1988) have been described. At small scales, various equipment exists for magnetic separations up to a scale of 50 ml, e.g., magnetic racks or fully automated robotic systems (Safařik & Safařiková, 2004). However, the original ideas of large-scale enzyme immobilisation and bioseparation have attracted less interest and have not yet been commercialised. Franzreb (2006) reported that it is only within the last decade that a focused effort has been made to develope scalable magnetic adsorbent-based bioprocesses. Moreover, small pilot scale examples of bio-purifications have only been reported within the last ~4 years (Ebner, 2006, Hollschuh & Schwämmle 2005). Thus, the studies done here represent the first results on the semi-continous multicycle re-use of immobilised lipases by HGMS in mini pilot scale.

5.2.1 Principle and properties of HGMS

A basic condition for the future use of HGMS processes in industry will be the need to recycle the magnetic adsorbents and the immobilised enzyme or lipase over many process cycles. The reusability will thus be dependent on the physical and chemical make-up as well as on the functionalisation chemistry employed. The idealised magnetic adsorbent for use in high gradient magnetic separation will briefly be described with the following idealised criteria (Franzreb *et al.,* 2006). The magnetic characteristics of the magnetic particle should be superparamagnetic and with a high magnetic susceptibility to ensure fast and efficient separation. The size should be between 0.5 and 2 µm to provide sufficient surface area (in the range of 20-100 m^2g^{-1}), and the particles should have the same size to guarantee that they all move with the same velocity in the magnetic field. The magnetic particles should be separated easily with the aid of a low-moderate strength magnetic field, and to achieve this, the saturation magnetisation should be 35 Am^2 kg^{-1} or greater, and the particles' size should be at least 500 nm. Further, the particles should be roughly sperical to achieve high adsorbent packing densities within magnetic filters. The particles should be robustly constructed to tolerate harsh chemical conditions during cleaning and regeneration and to provide a long life time for the supports. The surface design of

the particles should be nonporous to provide both fouling resistance and easier cleaning compared to porous particles. Furthermore, ligands can be utilised much more easily on nonporous particles and these particles most likely show higher stability against mechanical treatment. The surface should be neutrally charged, hydrophilic, and provide an easy-to-derivatise binding surface to allow ligands to couple at high densities and to avoid non-specific binding (Franzreb *et al.,* 2006).

Generally, magnetic separators are based on the property that magnetic fields exert a force on matter. The general relationship for this magnetic force, F_m is:

$$Fm = \mu_0 \, V_p \, M_p \, grad \, H \tag{2-1}$$

were μ_0 denotes the permeability constant of the vacuum, V_p the particle volume, M_p the particle magnetisation, and grad H the gradient of the magnetic field strength at the position of the particle. The magnetic force achievable in the separator can mainly be influenced by the prevailing field strengths and by its gradient, as the type of particles to be separated is usually given. High gradient magnetic separators (HGMS) achieve field strength of up to 1-2 T and gradients of up to about 10^5 T m^{-1} and thus are more powerful than other separators. Excellent explanations of magnetic separators and detailed mathematical descriptions can be found in text books, e.g., Gerber & Birss (1983) and Svoboda (1987).

Basically, the principle of HGMS is quite straight forward and can be described as in Franzreb (2006) whereby a canister filled with a magnetisable separation matrix is introduced into the area of an external magnetic field (Figure 7). The matrix may consist of a loose package of rough steel wool or of wire meshes stacked on top of each other. Further, the matrix wires bundle the external magnetic field in their surroundings to generate areas on their surface that strongly attract paramagnetic or ferromagnetic particles. The result is that the magnetic forces acting on the particles may exceed the weight acting on them by a factor of more than 100 (Franzreb *et al.,* 2006).

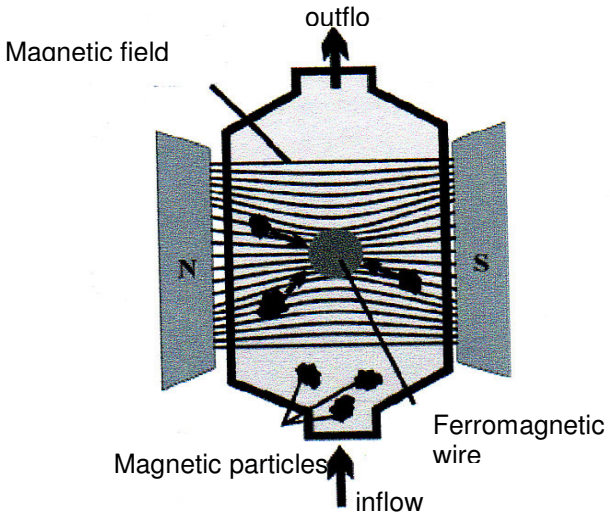

Figure 7: Basic principle of HGMS

Particles are trapped on the surface of the matrix, and the purified suspension flows out of the system. The shape of the area where particles are attracted is shown in figure 8 for a magnetic single wire. The area where particles are trapped generally is bigger then the dimension of the wire cross section. When the capacity of the cassette is reached, one has to stop the flux of suspension to avoid breakthrough of the particles. The washing of the particles from the cassette normaly is done by flushing the cassette in the direction contrary to the suspension flux entering the cassette. After that a new cycle of separation can be performed.

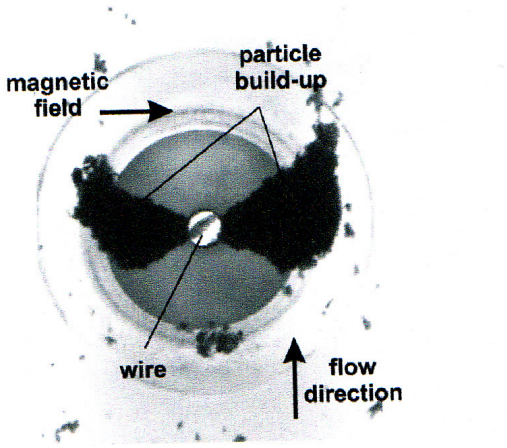

Figure 8: Areas of magnetic copper hydroxide flakes when trapped on a magnetisable single wire.

Generally, a HGMS system that is suitable for the efficient separation of magnetic adsorbent particles should be able to generate background magnetic fields of at least 0.3 T or more. Furthermore, it should show particle separation capacities on the level of 100 kg m^3 of matrix volume. Fields higher than ca. 1 T will allow higher flow velocities during separation, but magnetic fields beyond 1 T appear to offer no relevant benefits because the magnetic particles and the separation matrix material will be magnetically saturated, and thus the resulting magnetic forces will remain constant.

5.2.2 Geometric configuration of the HGMS cassette

In general, there are three different possible arrangements of suspension flux, wire and magnetic field, namely longitudinal, transversal and axial. The first arrangement (longitudinal) would represent the suspension flux running parallel to the magnetic field. The magnetic field would be perpendicular to the wire axis. The inducted magnetic poles of the wire thus can be found towards the flux directed side of the wire and towards the rejected side of the flux direction (Figure 9). In case of low Reynold's numbers, the particles are trapped exclusively on the current directed side. Furthermore HGMS filters, can have a transversal or axial geometric construction. In the case of a transversal construction, the suspension flux, the magnetic field, and the axis of the wire are perpendicular to each other. Particles are then trapped in two opposite areas. Those areas are located 90° from the stagnation point from the current directed side. In the case of an axial construction of the HGMS filter, the suspension flux is parallel to the wire axis. The particles are then trapped in the layer of the magnetic field and along the wire.

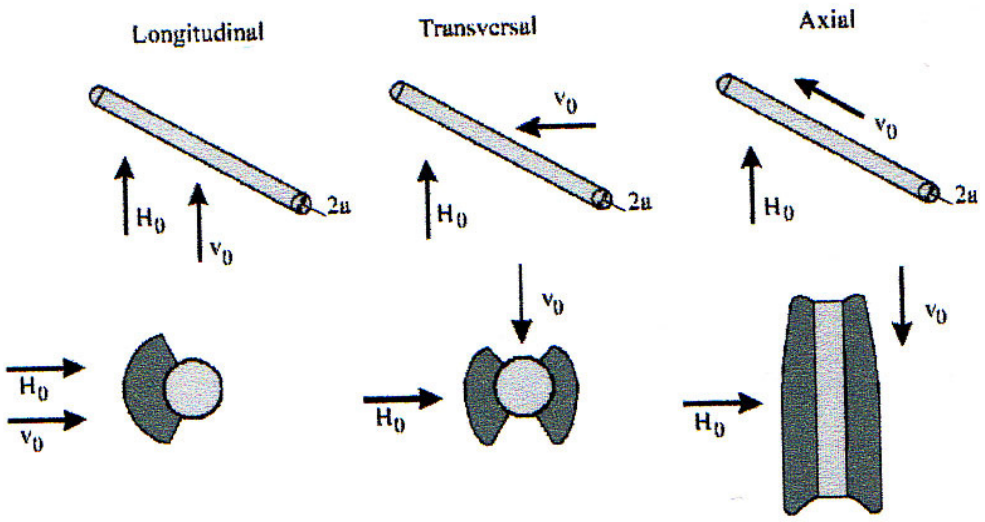

Figure 9: Geometric configurations for suspension flux, wire, and magnetic field, and the particle trapping.

Alternative magnetic separators to those based on HGMS that may be suitable for performing protein purification with magnetic adsorbent particles, at least at the pilot scale, are excellently reviewed and summarised in Franzreb (2006).

“ The pure and simple truth is rarely pure and never simple “

Oscar Wild

6 Development of a new basic spectrophotometric assay for online measurement of the activity of lipase immobilised on micro magnetic particles

Lipases (triacylglycerol hydrolases (EC 3.1.1.3)) act at aqueous and hydrophobic interfaces and lead to the breakdown of fats and oils. They are an important class of enzymes that are used in many processes, for example in the dairy industry (Nagao and Kito, 1990), the oil and fat industries (Mukherjee, 1990) and during the manufacture of detergents (Fujii *et al.,* 1986). Immobilisation of lipases allows repeated use of the enzyme by permitting separation of the product. Therefore continuous processes become possible and economically more attractive. However industrial use and multiple re-use of immobilised lipases in production is still limited due to their cost and the way they are used. Enzymes are typically immobilised on large porous particles and packed in columns or added batch wise to a reaction mixture and recovered by centrifugation. Such large porous particles suffer from diffusion limitations (Bozhinova *et al*., 2004), their separation is difficult if the reaction solution is dense or viscous and in columns, particulates must be avoided in the feed. All of these problems can potentially be overcome by using non porous magnetically susceptible immobilised lipases. Experimental development and application of lipases and their industrial use require a robust and reliable method for enzymatic activity measurement (for reviews see Kazlauskas and Bornscheuer *et al.,* 1998; Schmid and Verger, 1998). Furthermore, in this work, it was important to develope a reliable and rapid assay to permit evaluation of different enzyme immobilisation procedures.

Mosmuller (1992) showed that the major factor controlling lipase activity, in the case of substrate emulsion, is the amount of substrate molecules at the interface, which is proportional to the surface area of the emulsion. This characteristic of lipases makes it difficult to develope reliable assays for lipase activity. Mosmuller (1992) described four different assay groups for the detection of lipase activity: 1) by physical changes in the reaction system during ester hydrolysis, 2) by monitoring the disappearance of the substrate ester, 3) by determining the amount of liberated alcohol during ester

hydrolysis, and 4) by quantification of free fatty acid formed. Besides these four different assay groups, there is a fifth, which is based on interesterification units (IU). The method is based on the determination of an ester formed by a derivatisation pretreatment of an acid (or acyl donor) and an alcohol followed by gas chromatography or HPLC. The method is time consuming but can be used for enzyme characterization. For example Lee (1998) used this approach to define the interesterification activity of lipases in organic solvents which produced restructured or modified triacylglycerols to change the fatty acid composition, or their positional distribution in the glycerol molecule.

Spectrophotometric assays show certain advantages for screening of lipase activity, such as short reaction times, simple detection of product development, and are less laborious than the types mentioned above. Substrates based on *p*-nitrophenyl esters are widely used (Winkler and Stuckmann, 1978) although various alternatives are also described in the literature (Farooqui *et al.,* 1984). Furthermore the use of *p*-nitrophenol based substrates has been adapted to high-throughput-screening by Konarzycka-Bessler (2003) for measuring the synthesis activity of lipases and hydrolases in organic solvents. In that work the reaction was performed in a heterogeneous system composed of solid enzyme and liquid reactants, a combination that apparently did not disturb the fluorescence measurement of synthetic activity.

A number of other workers have also explored the possibility of direct measurement of immobilised enzyme activity. Mort (1973) introduced the feasibility of measuring the activity of enzyme covalently bound to a support. Those authors measured the activity of sepharose bound aldolase using a spectrophotometric method and did not report that the insoluble particles interfered with the measurement. Weliky (1969) proposed the idea to measure enzyme or protein content that was bound to carboxymethylcellulose using a modification of the Lowry method. Additionally Lasch (1975) developed a stirring device for photometers and used it for continuous kinetic studies on sepharose- bound leucine aminopeptidase and trypsine. Two decades

later Galunsky (1994) measured the activity of penicllin-G-amidase immobilised on particles in a spectrophotometric assay.

The above works raise the possibility of measuring the activity of magnetic support immobilised lipases directly in a spectrophotometric assay. Thus we examine here for the first time how the common *p*-nitrophenyl palmitate based spectrophotometric measurement of free lipases can be adapted to provide a reliable assay suitable for screening lipase activity after enzyme immobilisation on different functionalised magnetic particles.

6.1 Model systems for the development of the assay

Magnetic particles: Magnetic poly vinyl alcohol magnetic beads (M-PVA) that had been activated were obtained from Chemagen Biopolymer Technologie AG (Baesweiler, Germany) and according to the manufacturer all three types used contained 50-60 % magnetic material. Furthermore the density of functional groups as determined by the manufacturer were as follows: Epoxy-activated magnetic beads (M-PVA E02) had 200 µmol epoxide g^{-1} particles and were stored in acetone at 4 °C; amino-activated magnetic beads (M-PVA N12) had 310 µmol NH_2 g^{-1} particles and were stored in 1 mM EDTA in H_2O, pH 7; carboxy-activated magnetic beads (M-PVA C12) had 950 µmol COOH g^{-1} particles and were stored in H_2O. Laboratory made micro superparamagnetic silanised beads were produced as described previously by Hubbuch and Thomas (2002a).

Candida antarctica A-type lipase (CALA or Novozym L868) was purchased from Novozymes A/S (Bagsværd, Denmark). The stock solution of CALA (mw 45 kDa) had a protein concentration of 10 mg ml^{-1} as determined with the Bradford assay and an activity of 1100 U ml^{-1} was found, were one unit (U) is defined as the cleavage of *p*-NPP at a rate of 1 µmol min^{-1}. The resulting specific activity was thus measured to be 110 U mg^{-1} lipase. *Candida antarctica* B-type lipase (CALB) was purchased from Novozymes. The specific activity of CALB (mw 35 kDA) was measured to be 0.05 U mg^{-1} lipase. Thermomyces lanuginosus lipase (lipolase), mw 29 kDa, was a gift from Novozymes and the specific activity was measured to be 1.8 U mg^{-1} lipase. *p*-NPP,

n-hydroxysuccinimide (NHS) and n-(3-dimethylaminopropyl)-n´-ethylcarbodiimide (EDC) were obtained from Sigma-Aldrich (Steinheim, Germany) and all other chemicals were of analytical grade.

6.2 Performance of the assay and preparation of enzymes and particles for immobilisation

Activation of magnetic particles: The epoxy terminated M-PVA particles used did not need activation prior to immobilisation of the lipases, however the carboxy and amine terminated types were activated with NHS and glutaraldehyde, respectively. NHS-activation was performed as described in the literature (Hermanson *et al.,* 1992) with the following modifications. Briefly, 100 mg of M-PVA C12 beads were resuspended in 0.05 M HCl. The magnetic particles were separated and mixed with a solution of 133 mg EDC and 27 mg NHS in 2 ml of 0.05 M MES buffer (pH 5.5) for 40 min. The magnetic particles were separated and washed 5 times with MES buffer (pH 5.5). Glutaraldehyde activation of M-PVA N12 beads was performed using a method modified from the literature (Müller-Schulte, 2001) and was conducted just before the immobilisation of the enzyme. Briefly, 300 mg of M-PVA N12 beads were functionalised in 20 ml 0.1 M phosphate buffer (pH 7.0) containing 12.5 % glutaraldehyde. The reaction was performed for 2 h at 30°C in an overhead mixer (multifunctional mixer, Roto-Shake Genie, Roth, Karlsruhe). Lab-made silanised magnetic particles were coated with glutaraldehyde as previously described by Hubbuch and Thomas (2002a) before the immobilisation of the enzyme.

Immobilisation of the lipases: Immobilisation of all the lipases studied was conducted in the same way for all three activation methods examined and both commercial and lab-made magnetic particles. The activated particles to be used (60 – 300 mg) were washed with deionised water then with the buffer used for immobilisation (0.1 M sodium phosphate, pH 7) and resuspended in that buffer at a volume that would give a concentration of 30 mg ml^{-1} after addition of the enzyme. The lipase was then added to give the enzyme concentration required (see result section). The solution was incubated with an overhead mixer (multifunctional mixer, Roto-Shaker Genie, Roth Karlsruhe) over night at room temperature. Immobilisation was stopped by magnetic capture of the magnetic particles and washing.

Unreacted lipase was washed from the magnetic particles with buffers of different ionic strength and pH values. Washing was conducted consecutively for ~120 s with each of the following: 0.5 M potassium phosphate (pH 8.3) in a volume of 2 ml, 0.2 M an acetate buffer (pH 4.9) in 2 ml, a 0.1 M sodium phosphate buffer (pH 8) in a volume of 20 ml and twice with immobilisation buffer in a volume of 20 ml. The supernatant after immobilisation as well as the washing fractions were collected and analysed for specific activity and protein content.

Activity assay for free and immobilised lipase: The spectrophotometric assay was based on the ability of lipases to cleave *p*-nitrophenyl palmitate (*p*-NPP) thus releasing a stoichiometric amount of *p*-nitrophenol (*p*-NP) (Winkler and Stuckmann, 1978), which is an intensively coloured yellow compound that can be measured by absorbance at a wavelength of 410 nm (A_{410}). A 10-200 mM *p*-NPP-stock solution in acetone was first prepared and 10 µl added to 940 µl of measuring buffer (0.1 M sodium phosphate, 10 % v/v acetone, 4 % v/v triton x-100, 0.2 % w/v gum arabic, pH 8). The solution was incubated at 60 °C in a waterbath (B.Braun, Biotech international, Thermomix ME) for 300 s then cooled to 29 °C (over ~300 s). Subsequently the reaction was started by adding this solution to a prewarmed (29 °C) cuvette containing 50 µl of the lipase solution (free or immobilised) to be tested and mixed once for 1 s with a spatula then placed immediately in a temperature controlled spectrophotometer (Ultraspec 2100 pro UV/Vis AmershamBioscience, Freiburg, Germany). No further mixing was used unless stated in the text. Since *p*-NPP could only be dissolved in the phosphate buffer up to a concentration of 2 mM it was not possible to study the complete kinetic parameters of the lipase. It was found that magnetic particles did not cleave *p*-NPP in the absence of free or immobilised lipase. The volume activity (VA) of the free and immobilised lipase was calculated according to equation 3. One unit of activity (U) is defined as the cleavage of *p*-NPP at a rate of 1 µmol min^{-1} (Figure 10).

$$VA = \frac{\Delta A_{410} \quad V_s}{V_{ls} \quad L \quad \varepsilon_{410}} \qquad Equation \ 3$$

Here ΔA_{410} is the rate of change of absorbance at 410 nm min^{-1} . V_s is the total volume of the reaction mixture. V_{ls} is the volume of the sample containing the lipase to be measured. L is the path length of the cuvette. The extinction coefficient ε of *p*-NPP depends on the type of buffer, pH and temperature. For the conditions used here, the extinction coefficient at 410 nm (ε_{410}) was found to be 13.5 at 29 °C in the buffer used for the lipase activity measurement.

Figure 10: Device used for spectrophotometric assay for the direct determination of micro magnetic particle immobilised lipase activity.

Total protein measurement: The concentration of protein in samples was determined using the Bradford assay with reagents from Biorad (Munich, Germany) and was conducted according to the manufacturers recommendations. Lipase concentrations were determined using a standard curve with bovine serum albumin (BSA).

pH-stat activity assay for free and immobilised lipase: A pH-stat system (Radiometer A/S, Copenhagen, Denmark) was used consisting of a PHM 63 digital pH meter with a Metrohm electrode (Unitrode,6.0259.100), TTA 60 titration assembly, a TTT 60 titrator and an autoburette, Model ABU 12. A 25 ml reaction vessel was filled with *p*-NPP emulsified in buffer (0.1 M sodium phosphate buffer pH 8 containing, 10 % v/v acetone, 4 % v/v triton X-100 and 0.2 % w/v gum arabic) and mixed with an overhead

stirrer. The immobilised enzyme was then added and the amount of 1 M NaOH needed to hold the pH constant at 8 was recorded with a servograph recorder (Radiometer, Denmark). The temperature in the reaction vessel was maintained at 40°C by a Lauda K-2/RD circulation water bath (Brinkmann Instruments, Inc., Westbury, NY 11590).

6.3 Results of systematic study to proove the validity of the new assay method

The *p*-nitrophenol ester assay was chosen to demonstrate the feasibility of directly measuring the activity of immobilised lipases on magnetic particles using a spectrophotometer.

6.3.1 Comparison of the absorption spectrum of the particles and of p-NP

The absorption spectrum of the cleavage product *p*-nitrophenol was measured by scanning in the range of 200 nm – 900 nm. The absorption maximum was found to be at 410 nm which is consistent with previous reports in the literature (Winkler and Stuckmann, 1978). The absorption maximum of M-PVA E02, M-PVA C12 (with and without activation by NHS) and M-PVA N12 (with and without activation by glutaraldehyde) was also determined, and was found to be at wavelengths above 500 nm, in general. Therefore, it was concluded that absorbance measurements at a wavelength of 410 nm would reflect the concentration of *p*-NP specifically.

6.3.2 Effect of the amount of particles in the cuvette on the assay

Since high magnetic particle concentration in the reaction could lead to turbid conditions, the A410 of a range of M-PVA C12 concentrations was determined. M-PVA C12 concentrations from 0.01 mg ml^{-1} to 0.5 mg ml^{-1} were examined in the presence of approximately 1.6 mg ml^{-1} (ca. 183 U ml^{-1}) free CALA and 1 mM *p*-NPP. It was determined that magnetic particle concentrations over 0.2 mg ml^{-1} interfered with the assay. Therefore, the final immobilised enzyme particle concentration in the spectrophotometer cuvette was always below 0.2 mg ml^{-1} in all further work.

6.3.3 The effect of stirring on the assay

To study the possible effect of particle settling, as well as mass transport and diffusional limitations during the assay, analysis with and without stirring during measurement was conducted in cuvettes containing immobilised lipase or free enzyme. The activity of CALA immobilised on M-PVA C12 particles was measured to be 60 U g^{-1} particles under a set of 'standard' conditions, i.e. ~ 0.15 mg ml^{-1} immobilised enzyme and 1 mM *p*-NPP in the cuvette. In contrast, the specific activity of the free enzyme (10 mg ml^{-1}) with 1 mM *p*-NPP was found to be 90 U mg^{-1}. No effect of stirring on measured enzyme activity was found (Figure 11). Therefore, it was concluded that stirring during the assay was not necessary for the immobilised or free enzyme (Figure 11).

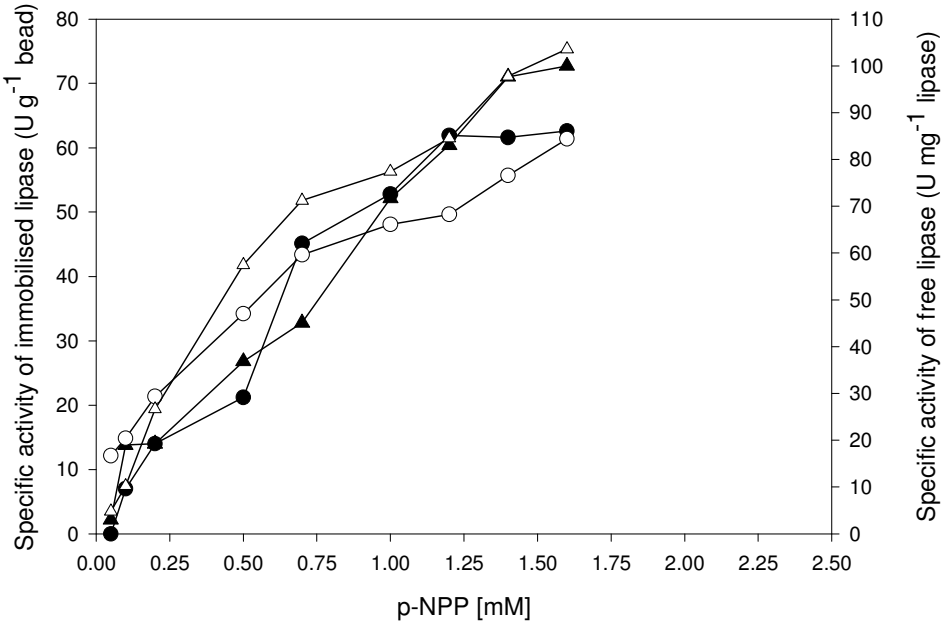

Figure 11: Effect of stirring during spectrophotometric measurement of free CALA and CALA immobilised on NHS-activated carboxy M-PVA beads. Stirred immobilised lipase (▲); unstirred immobilsed lipase (●); stirred free lipase (Δ); unstirred free lipase (○).

The magnetic particles (1-2 µm) tend to settle down in the solution over extended periods of time (e.g. overnight). However, in the time interval of the assay (120 s), no particle-settling effects on the assay were observed. This was examined using 0.13 mg ml^{-1} particles (without any free or immobilised enzyme) in the cuvette, and no interference by the particles on the absorbance over 120 s was seen (Figure 12). In contrast, immobilised CALA with a specific activity of 60 U g^{-1} particles resulted in an absorbance change of ~0.3 over the background during a 120 s period (Figure 12). Furthermore when the equivalent amount of free CALA (i.e. 0.01 U ml^{-1}) was incubated with 0.13 mg ml^{-1} naked particles (i.e. without lipase immobilised) the same adsorbance change of 0.3 was observed in 120 s. Based on these results, it was concluded that reliable measurements can be made when the ΔA_{410} is at least 0.1 min^{-1} to ensure that magnetic particle settling does not interfere in the assay (Figure 12).

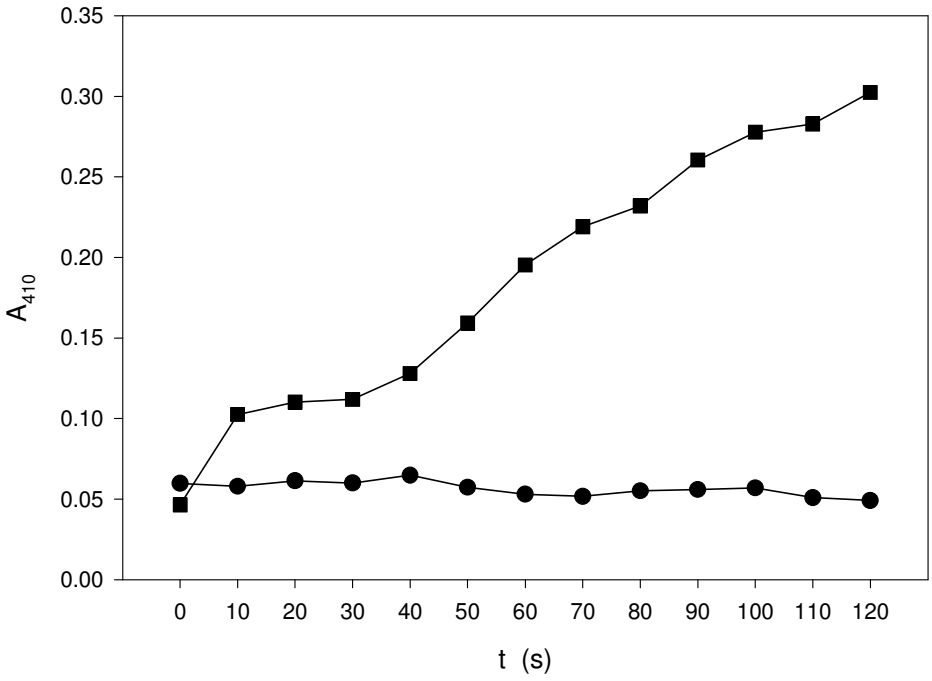

Figure 12: Influence of the particles on the adsorbance change due to settling of the beads in the cuvette of the spectrophotometer (ΔA_{410} = -0.8 x 10^{-3} min^{-1}) (●) compared with ΔA_{410} resulting from substrate cleavage by magnetic particles immobilised with CALA (ΔA_{410} =~0.1 min^{-1}) (■).

6.3.4 Bead related specific activity of the immobilised lipases (U mg^{-1} bead) at different concentrations

In light of the above results, the influence of different concentrations of immobilised enzyme on the assay was examined in order to test if it would yield a constant bead related specific activity. The results in figure 13 show that the bead related specific activity was constant at ~0.580 U mg bead^{-1} as the concentration of epoxy activated magnetic beads with CALA immobilised was increased. Thus, the total lipase activity in solution increased proportionately with increasing immobilised enzyme concentration, since although we are working below the k_m for the free enzyme, the reaction rate is expected to be linearly dependant on enzyme concentration for a fixed concentration of substrate.

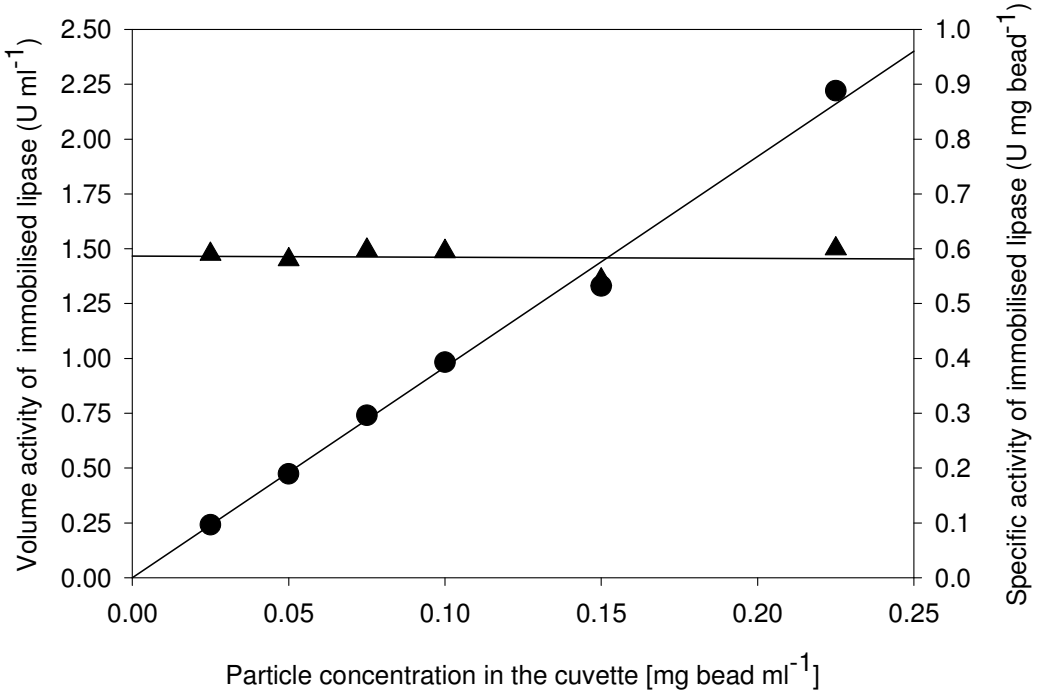

Figure 13: Effect of different concentrations of magnetic bead immobilised enzyme on the activity measured (●). Normalisation of the activity measured to the amount of immobilised enzyme used (▲).

6.3.5 Validation of the assay

The spectrophotometric assay developed here was validated against measurements with a pH-stat. The pH-stat was used to titrate the acidic products formed from *p*-NPP cleavage by immobilised CALA. First, two reactions were set up in parallel, one in which 1 mM *p*-NPP cleavage by 0.15 mg ml^{-1} immobilised enzyme was followed with the spectrophotometric method and one with the pH-stat. The amount of *p*-NP liberated after each minute was calculated for a period of 10 minutes. The results are plotted in figure 14 and it can be seen that a close relationship exists between the two methods. By plotting the data in figure 14 against each other, a linear correlation of $y = 3.9x$ and $r^2 = 0.97$ was found using a least squares linear regression with SigmaPlot (Systat Software), where y equals the amount of *p*-NP formed in the spectrophotometric assay and x equals the amount of *p*-NP formed in pH-stat assay. In the second validation, the initial rate of *p*-NPP cleavage resulting from different concentrations of immobilised CALA (0.0075 to 0.045 mg ml^{-1}) was measured by the spectrophotometric assay and compared to that from the titrator. A linear correlation with the relationship $y = 1.7x$ and $r^2 = 0.95$ was found, where x and y are the same as described above.

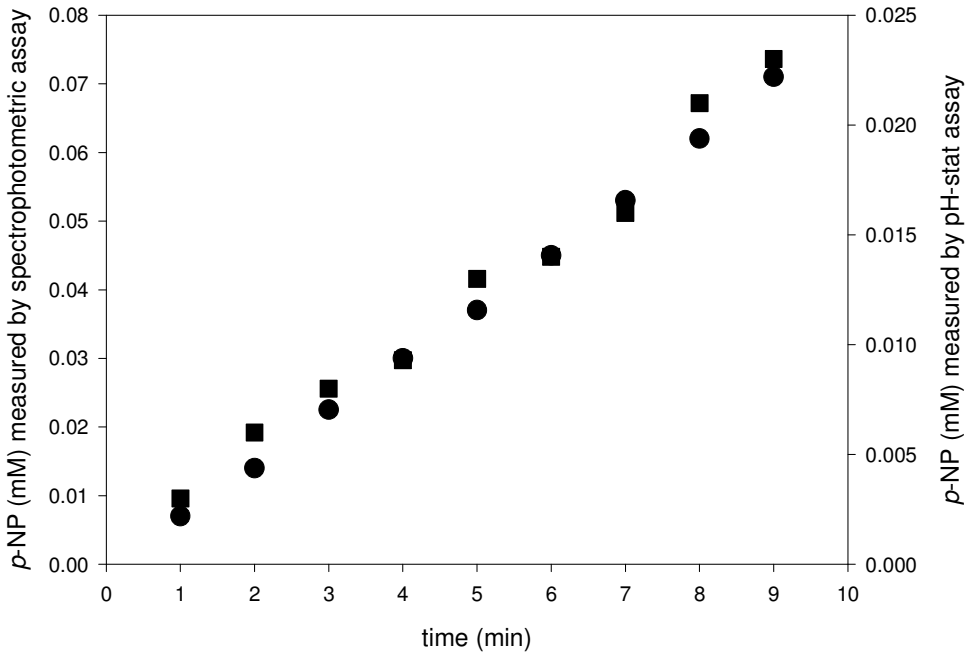

Figure 14: Direct comparison of the spectrophotometric assay (●) with a pH-stat method (■) for measuring product development by magnetic bead immobilised CALA. Measurement of the amount of *p*-NP formed from cleavage of 1 mM *p*-NPP after 1 minute and each minute thereafter for 9 minutes.

6.3.6 Generic application of the bead related specific activity (U g^{-1} bead) assay

CALA was immobilised on M-PVA E02, N12 and lab-made particles (30 mg ml^{-1}). Table 6 compares the bead related specific activities of CALA immobilised on those different types of magnetic particles and activation chemistries. It was observed that CALA immobilised on M-PVA E02 resulted in the highest bead related specific activity. CALA immobilised on lab-made silanised particles showed the next best bead related specific activity followed by CALA immobilised on M-PVA N12. In addition, it was found that the bead related specific activity of CALA immobilised on lab-made silanised particles could be doubled (687 U g bead $^{-1}$) by doubling the amount of enzyme offered during immobilisation.

The immobilisation of lipolase and CALB was also conducted and the determination of their bead related specific activities was possible using the spectrophotometric

method developed here. Lipolase immobilised on M-PVA E02 resulted in a bead related specific activity of 69 U g^{-1} bead using 0.8 mg ml^{-1} protein for the immobilisation reaction. CALB immobilised on M-PVA N12 showed the lowest bead related specific activities of ~40 U g^{-1} bead.

Table 6: *Comparison of the bead related* specific activity of CALA immobilised on different types of magnetic particles.

Magnetic particle used [a]	*Lipase type*	*Initial concentration of free lipase before immobilisation* (*mg protein per ml^{-1} of reaction solution*)	*Initial activity of free lipase in coupling solution* (*U ml^{-1}*)	*Amount of lipase coupled* (*mg g bead^{-1}*)	*Bead related immobilised enzyme activity* (*U g bead^{-1}*)
M-PVA, epoxy	CALA	0.22	25	4	733
lab made, silanised	CALA	0.22	25	7	347
M-PVA, amino	CALA	0.2	25	4.3	233

6.4 Potential of the newly developed assay for screening of immobilised lipases

In this work we have demonstrated that the *p*-nitrophenyl ester assay can be used to constantly and directly measure the activity of magnetic bead immobilised lipases via a change of adsorbance. The complete assay can be performed very rapidly within ~4 min per sample including sample preparation (i.e. dilution and pipetting into cuvettes) and measurement time. The only specialised equipment needed is a conventional spectrophotometer. Of great benefit is the finding that magnetic separation of the immobilised lipase is not necessary before measurement. This stands in contrast to the end point determination used by Huang *et al*., (2003) and

Pancreac´h *et al.,* (1997) in which immobilised lipase was removed from the assay solution before measuring the absorbance of *p*-NP at 410 nm. The assay presented here is thus much faster and easier than alternative methods and requires little immobilised enzyme or substrate.

The method developed here can be expected to be useful for fast screening of immobilisation chemistries for other enzymes and could probably be implemented in 96 well microtiter plate format and with robotic spectrophotometers. The rapidness of the assay, and reduction in handling steps (i.e. no removal of immobilised enzyme or stirring required during measurement) may provide an opportunity for complete automisation of the method, e.g. via flow injection analysis to permit online measuring of activity in HGMF based industrial processes, which will require monitoring of the enzyme activity during the process and before re-use.

6.5 Summary of the development of the basic assay system

A spectrophotometric assay has been adapted to directly measure the activity of enzymes immobilised on insoluble magnetic particles. Three different types of lipases (*Candida antarctica* lipase A and B as well as *Thermocatenulatus lanuginosus* lipase) were immobilised on two types of magnetic beads. The activity of the resulting immobilised lipase preparations was measured directly in the reaction solution by using a modified *p*-nitrophenol ester assay using a spectrophotometer. Removal of the solid particles was not necessary prior to spectrophotometric measurement, thus allowing reliable kinetic measurements to be made rapidly. The method was found to be effective for a wide range of magnetic bead concentrations (0.01 – 0.2 mg ml^{-1}). In all cases the assay could be used to determine the bead related specific enzyme activity. The assay was validated by comparing with a pH-stat method using *p*-nitrophenol palmitate as the substrate and we found excellent correlation between the two methods. The utility of the spectrophotometric assay was demonstrated by applying it to identify the best combination of lipase type, activation chemistry and magnetic particle. It was found that epoxy activation of poly vinyl alcohol (PVA) coated magnetic particles prior to immobilisation of commercial *Candida antarctica* lipase A (CALA) gave the best preparation.

7 Immobilisation of *Candida antarctica* on micro magnetic particles: Zeta-potential measurement as a diagnostic tool in enzyme immobilisation

Enzyme immobilisation has several advantages such as easy separation of the product, and its recovery, repeated usability of the enzyme, reduced costs, etc. Therefore continuous processes with immobilised enzymes become economically viable. Enzymes are typically immobilised on large porous particles which are either packed in columns or added batch wise to a reaction mixture and are recovered by centrifugation for further use. This process has potential problems such as diffusion limitations of the substrate (Bozhinova *et al.,* 2004) difficulty of separation of the particles from viscous reaction solutions. Moreover, porous particles also could be easily “plugged” with reaction components, particularly in complex industrial mixtures. Some of these problems could be partially overcome with using non-porous matrix support.

Currently, the non-porous particles are commonly amino-activated, epoxy-activated or carboxy-activated. The covalent nature of activation makes these particles very stable for enzyme immobilisation. The affinity of an enzyme to a specific kind of activated non-porous particle not only depends on the nature of chemical interactions (Ghazi *et al.,* 2005 and Klibanov 1983 and Mateo *et al.,* 2005) but also on the electrostatic interactions between them (Grüttner 2002 and Sano *et al.,* 1993 and Hubbuch 2002b). When the activated non-porous particles are suspended in a solution, they develop an electric charge due to their ionic characteristics, and each carrier particle is surrounded by a fixed layer of oppositely charged ions (Stern layer), which is enveloped by a diffuse layer with ions of different polarities (Figure 15). The electric potential in the diffuse layer decreases exponentially to zero. The electric potential at the shear level in the diffuse layer is known as the zeta-potential. Despite the important role of the zeta-potential in enzyme immobilisation, there are very few reports considering its measurement before immobilising the enzyme onto the particle. This concept has been previously demonstrated in the binding of bovine serum albumin to micro particles (Grüttner, 2002). The influence of the zeta-potential

of colloidal Al_2O_3 particles upon the adsorption capacity of bovine serum albumin was investigated (Rezwan *et al.,* 2004). The change of the isoelectric point (IEP) of the mixture of Al_2O_3 and bovine serum albumin, was found to be a function of the mass of coupled protein (Rezwan *et al.,* 2004).

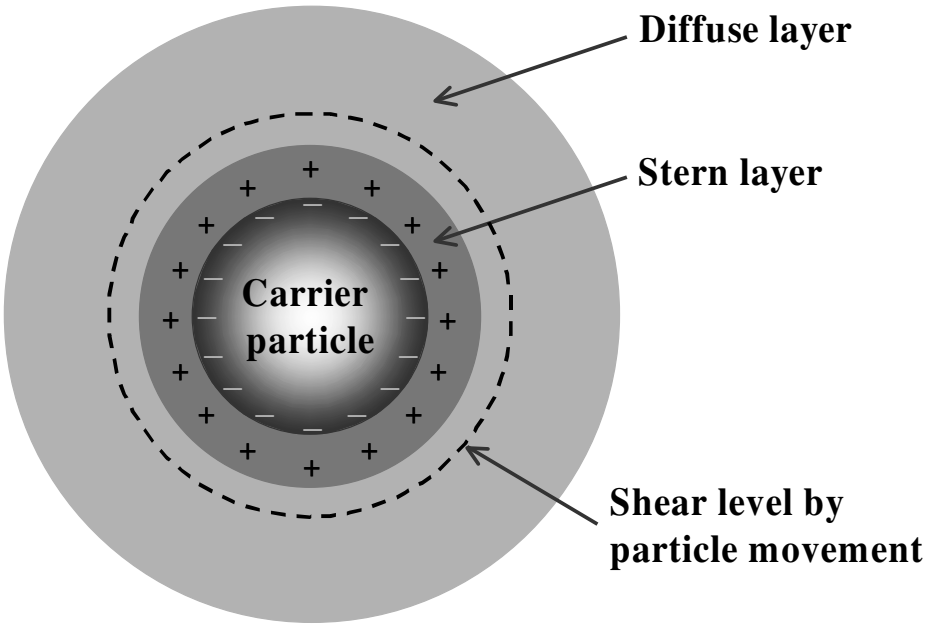

Figure 15: Depiction of the zeta-potential of a negatively charged particle. The particle is surrounded by a fixed Stern layer of oppositely charged ions (in this case, positive ions) and outside this layer is a diffuse layer with ions of different polarities. The electric potential in the diffuse layer decreases exponentially. The potential at the shear level in the diffuse layer is known as the zeta-potential and as this potential approaches to zero, the particles tend to aggregate.

In order to further demonstrate the importance of zeta-potential, *Candida antarctica* A-type lipase (CALA) was used as a model protein for immobilisation on non-porous magnetic micro particles with three different terminations (epoxy, carboxy and amine) and measured the zeta-potential in each case. The goal of this work is to establish a correlation between the zeta-potential of the protein and the particle with their binding affinities.

7.1 Measurement of the size and the zeta-potential of the magnetic particles and the lipase of *Candida antaractica*

Ultracentrifugation: Removal of insoluble particles was done by ultracentrifugation (Optima TLX, Beckman Coulter) of BSA and CALA at 35000 rpm (60000 × g) for 3 hours. Centrifuge tubes were filled with 3.5 ml of the protein solution. The purified protein was collected immediately after the centrifuge stopped. 1.5 ml of the supernatant of the samples was retained from each tube by a pipette and saved at 4 ℃ for further examinations.

Determination of size and zeta-potential of the particles and the proteins: In the protein solutions, the particle size distribution as well as the zeta-potential was measured before and after ultracentrifugation with the help of a Zetasizer Nano ZS (Malvern Instruments). The particle size distribution was measured by dynamic light scattering (scattering angle: 173°). For the zeta-potential measurements, the laser doppler electrophoresis technique was used. The instrument calculated the zeta-potential from the electrophoretic mobility using the Smoluchowski equation (Equation 4).

$$\xi = \frac{U\eta}{\varepsilon} \quad \textit{Equation 4}$$

where:

ζ zeta-potential

U electrophoretic mobility,

η medium viscosity,

ε dielectric constant.

Additionally, the measurements of the particle size and zeta-potential of magnetic particles were carried out before and after CALA immobilisation. For the measurements a folded capillary cell (DTS 1060, Malvern Instruments Ltd) was used. For all measurements, the concentration of the particles was 0.15 mg ml^{-1} and the concentration of the CALA 0.15 mg ml^{-1}.

The effect of the pH value on the zeta-potential, the size of the magnetic particles and the activity of CALA was examined in distilled water. The samples imported enough salts to enable pH adjustment in distilled water. The titration of magnetic particles was carried out using an autotitrator (MPT-2, Malvern Instruments Ltd). The titration device was equipped with a sample container, which was connected through a capillary system, and with a peristaltic pump with a folded capillary cell. The samples (10 ml) containing magnetic particles were titrated from pH value 6 to 2 and from 6 to 11 using 0.1 M and 1 M hydrochloric acid and 0.1 M sodium hydroxide, respectively. After each pH adjustment, the particle size and the zeta-potential were detected. The samples were circulated and stirred during the pH adjustment.

Further information on zeta-potential measurements and titration experiments is given in Frimmel (2007). Furthermore, the centrifuged CALA was titrated in the range of pH 2 to 11. The pH value had to be manually adjusted as CALA was denaturated and precipitated by pumping through the tubes of the autotitrator that have a very small diameter (0.5 mm). The pH value was adjusted using the same solutions as for the pH adjustment of the magnetic particles. Furthermore, CALA (after ultracentrifugation) was pipeted with cut tips to possibly avoid denaturating of CALA by shear forces. After each pH adjustment, the particle size, the zeta-potential, and the activity of proteins were measured.

7.2 Systematic comparison of widely used matrix particles for enzyme immobilisation by means of zeta-potential measurement

In order to establish the impact of zeta-potential on the interaction of matrix and the enzyme during immobilisation, CALA (*Candida antarctica* A-type Lipase) was used as a model protein for immobilisation on non-porous magnetic micro particles with epoxy (M-PVA E02), carboxy (M-PVA C12) or amine (M-PVA N12) terminations. Since insoluble contaminants in the commercial CALA solution (Figure 16A) were observed which could potentially interfere with the zeta-potential measurements these contaminants were first removed using ultracentrifugation.

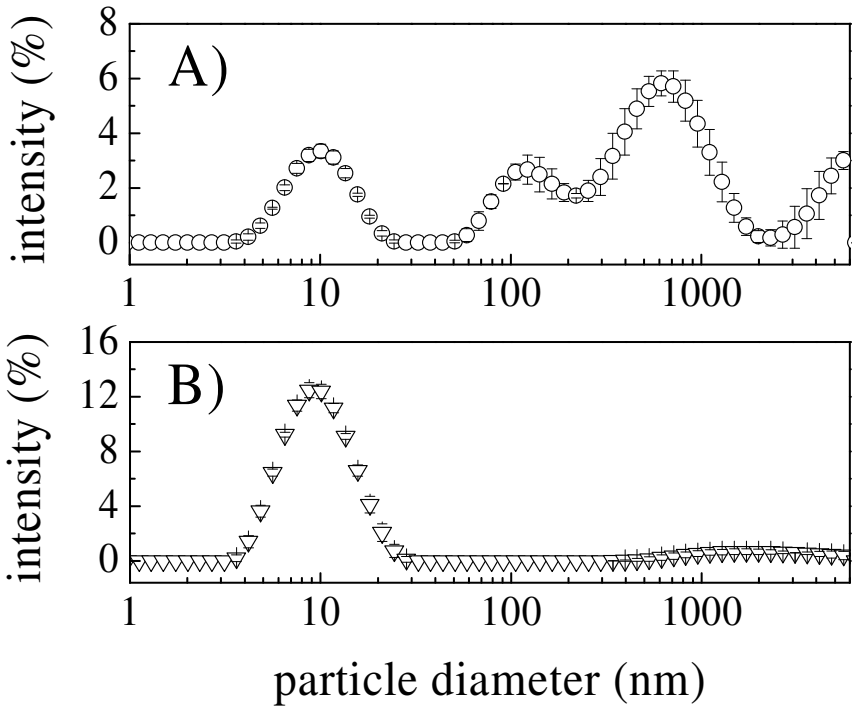

Figure 16: Removal of insoluble particles from CALA stock solution by ultracentrifugation. The stock solution (A) was found to contain insoluble particles which interfered with the assay. These were removed by ultracentrifugation (B).

After ultracentrifugation, a single peak for CALA solution, was observed as indicated by the particle size distribution (Figure 16B). The zeta-potential of CALA (after ultracentrifugation) in phosphate buffer and in distilled water was -8.2 mV and -8.7 mV respectively (Table 7).

Table 7: Zeta-potential and the size of CALA and different functionalised magnetic micro particles with and without immobilised CALA.

sample	Medium	specific activity (U g^{-1}) bead	amount of protein coupled (%)	Size (µm)	zeta-potential (mV)
CALA	Buffer	-	-	$9.7 \cdot 10^{-3}$	-8.2
CALA	DI water	-	-	$8 \cdot 10^{-3}$	-8.7
M-PVA E02	Buffer	-	-	4.15	-11.4
M-PVA E02	DI water	-	-	3.14	-23.2
M-PVA E02 + CALA	Buffer	430	29	3.44	-9.5
M-PVA N12	Buffer	-	-	5.76	+0.6
M-PVA N12	DI water	-	-	4.07	+17.7
M-PVA N12 + CALA	Buffer	213	76	3.82	-13.1
M-PVA C12	Buffer	-	-	3.29	-35.0
M-PVA C12	DI water	-	-	3.23	-54.6
M-PVA C12 +CALA	Buffer	33	15	4.84	-33.0

Buffer: NaH_2PO_4+Na_2HPO_4, c=0.1 M, pH 7
DI: solved in distilled water

7.2.1 Effect of the terminal group on CALA immobilisation

Next the impact of the three terminal groups on immobilisation of CALA were studied. In order to test the effect of the three terminal groups on the magnetic particles on the immobilisation characteristics, the coupling of CALA to the beads was measured. The bead related specific activity (total amount of activity in U divided by the amount of particles used for immobilisation) was compared with the amount of protein coupled (amount of CALA bound to the particle divided by the amount of protein of the stock solution) on the bead. Different bead related specific activities (U g^{-1} bead) were observed although the bead (30 mg ml^{-1}) to CALA (0.1 mg ml^{-1} and 1.0 mg ml^{-1}) ratio remained constant (Figure 17). With the three terminal groups on the magnetic beads, a reduction in the CALA coupling (%) was observed while the specific activity increased as the protein concentration increased. The amino-terminated beads (M-PVA N12) retained maximum amount of coupling (Figure 17B) compared with either epoxy-terminated (M-PVA E02, Figure 17A) or carboxy-terminated beads (M-PVA C12, Figure 17C). On the other hand, epoxy-terminated (M-PVA E02) beads resulted in a 2-4 fold higher specific CALA activity (Figure 17A).

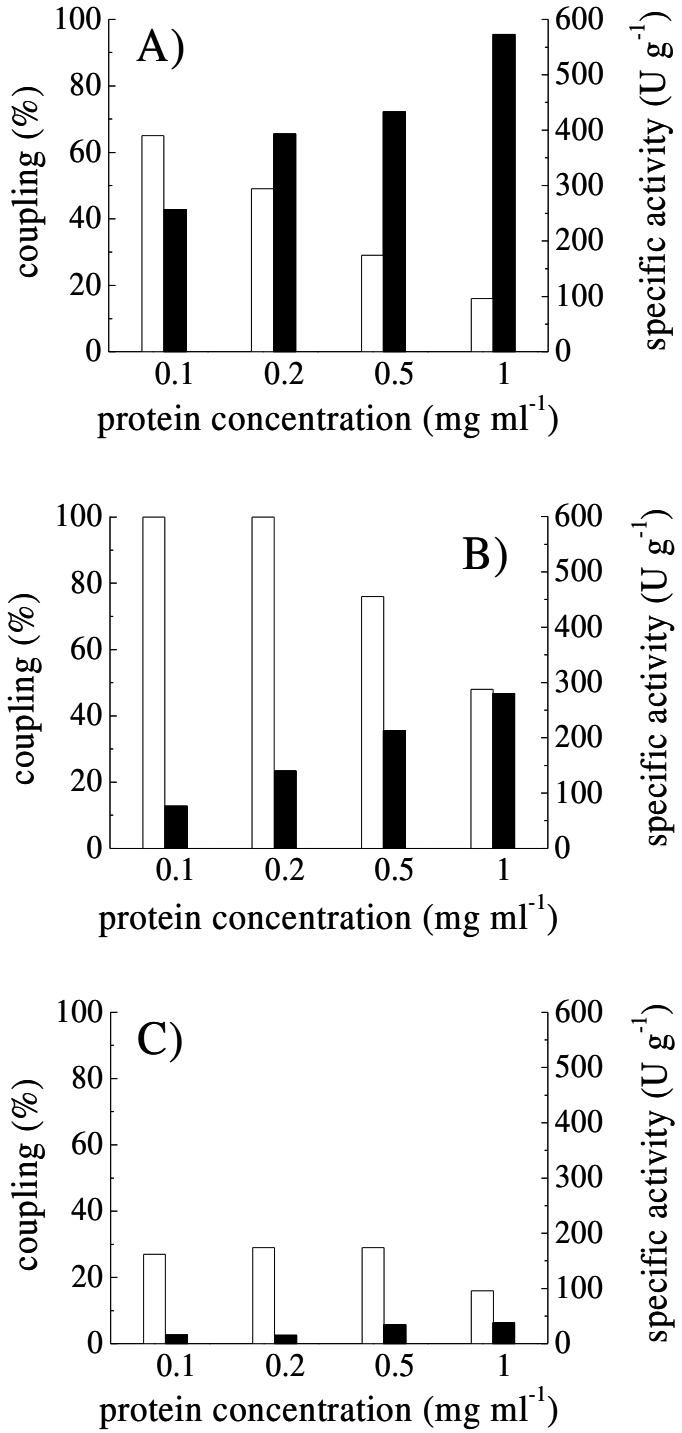

Figure 17: CALA immobilised on 30 mg ml-1 of (A) M-PVA E02 , (B) M-PVA N12, or (C) M-PVA C12 magnetic micro particles. The amount of protein coupled (□) and the specific activity (■) were measured as a function of protein concentration.

7.2.2 Zeta-potential determination of free CALA and magnetic micro particles

The zeta-potential, the size of CALA, and its specific activity as a function of the pH were measured. The zeta-potential consistently decreased with increasing pH, starting with +13 mV at a pH of 3 to -25 mV at a pH of 11 (Figure 18). The isoelectric point (IEP) of CALA (the pH where the zeta-potential is 0 mV) was observed at pH of 4 (Figure 18A). The size of CALA remained relatively constant at 10^3 nm between pH of 3 and 4, reaching a minimum of 10 nm at a pH of 5 before increasing to 10^2 nm at a pH of 11 (Figure 18B). At pH 4, CALA agglomerates to a size of about 10^3 nm. The specific activity remained relatively stable (about 120 U g^{-1}) in the pH range of 3 to 9, beyond which the activity decreased rapidly (Figure 18C).

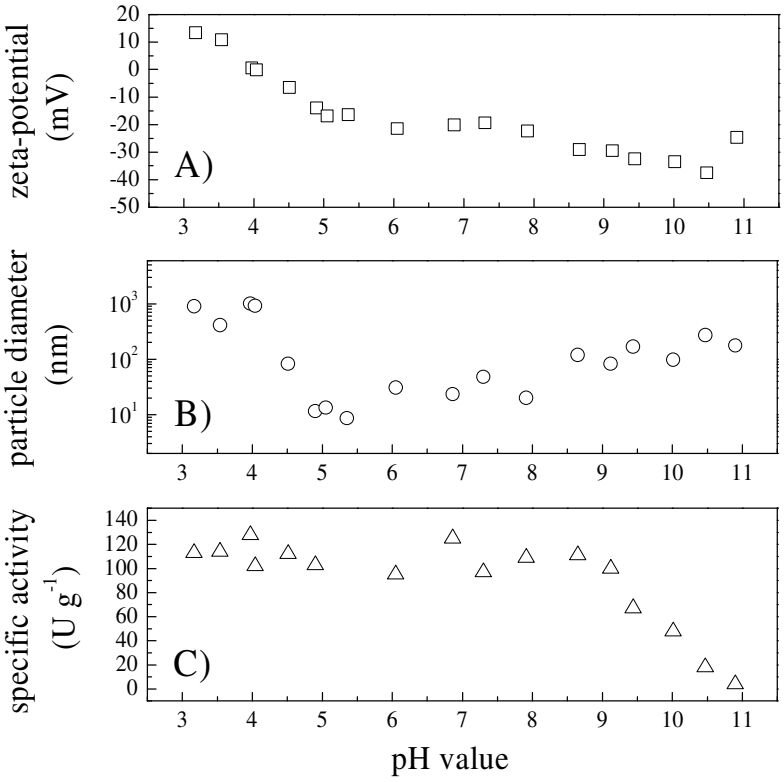

Figure 18: Zeta-potential (A), size (B), and specific activity (C) of free CALA as a function of pH.

Next, the zeta-potential and the size of the M-PVA N12 and the M-PVA E02 beads (without CALA) in distilled water as a function of pH value were measured. The zeta-potential of M-PVA E02 beads remained negative in the pH range 4 to 11. At lower

pH values (2 to 4), the zeta-potential was approximately 0 mV (Figure 19A). The particle size stayed constant over the entire pH range measured. In contrast to the observations on the M-PVA E02 beads, the M-PVA N12 beads showed a positive zeta-potential in the pH range of 2 to 7. The IEP was determined to be approximately at the pH value of 7.2 (Figure 19A). The strongest agglomeration of the particles was observed at the respective IEP values (Figure 19B).

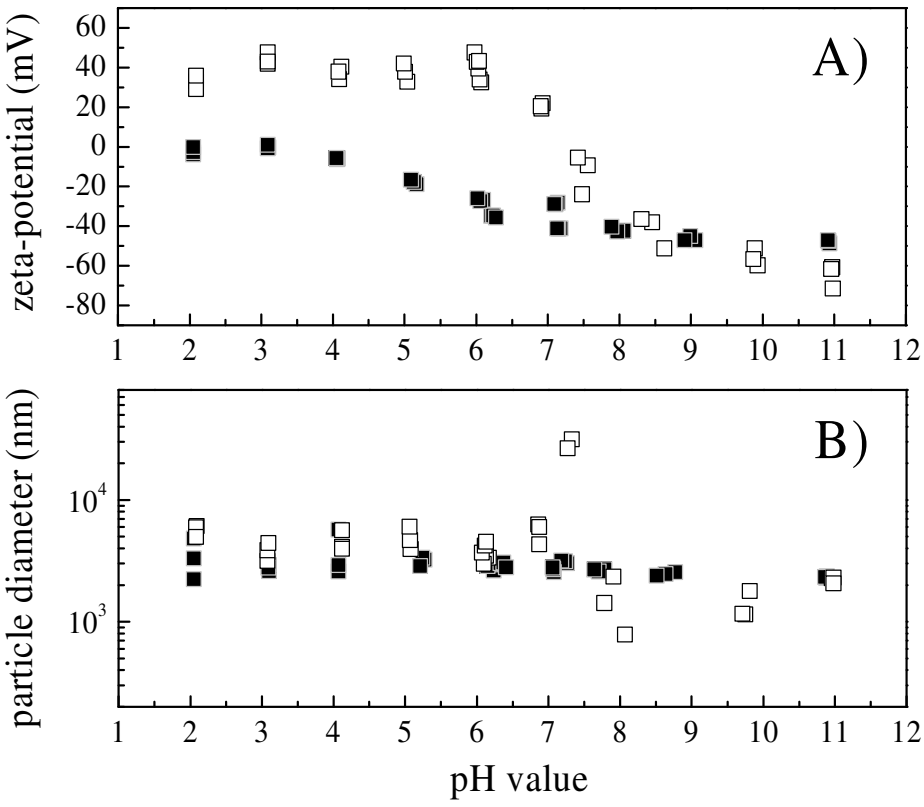

Figure 19: Zeta-potential (A) and the size (B) of M-PVA N12 (□) and M-PVA E02 particles (■) as a function of pH.

The zeta-potential and the size of the micro magnetic particles carrying immobilised CALA (Table 7) was also measured. M-PVA E02 beads had a zeta-potential of -11 mV in sodium phosphate buffer (pH 7) and a much lower value of -23 mV in distilled water. The amino terminated particles had a zeta-potential of +18 mV in distilled water. Agglomeration and consequently particle size enhancement was observed in

the phosphate buffer near the IEP (ζ = +0.6 mV). M-PVA C12 beads by far showed the most negative zeta-potential (about -55 mV) in distilled water, but only -35 mV in phosphate buffer (Table 7).

7.2.3 Influence of the pH value and the buffer concentration on specific activity of immobilised CALA

The influence of the buffer concentration and the pH on the specific activity of CALA was tested. The influence of the electrostatic interaction on the amount of CALA coupled onto the M-PVA E02 beads was investigated by varying the pH and the buffer concentration. CALA was immobilised in distilled water without washing the particles. Any significant difference in specific activity of immobilised CALA between pH 6 – 7 (Table 8) were not observed. Next, pH was set at 7 and the specific activity of immobilised CALA at different buffer concentrations was measured. The specific activity increased with the buffer concentration and reached a maximum of 933 U g^{-1} bead at 0.3 M phosphate buffer before it started decreasing (Table 8).

Table 8: Influence of pH value and puffer concentration on the specific activity of the lipase immobilised on M-PVA E02 beads.

pH	Activity (U g^{-1})
6	563
7	633
8	613
concentration of buffer (M)	
0	400
0.1	833
0.2	900
0.3	933
0.4	867
0.5	667

7.3 Potential of the optimised immobilised lipase *(Candida antarctica)* for feasibility studies of multiple recycling by magnetic separation technology

Despite the importance of zeta-potential for enzyme immobilisation (Grüttner 2002 and Rezwan *et al.,* 2004) a detailed correlation of the electrostatic interactions between the enzyme and the particle on their binding remains obscure. In this work, the influence of the zeta-potential on the adsorption process of immobilised lipase

from *Candida antarctica* type-A (CALA) on a type of micro magnetic particles (1µm) with three different terminations (epoxy, amino or carboxy) was investigated. The important role of zeta-potential in enzyme immobilisation and evaluation of the zeta-potential as a potential tool for selecting the optimum matrix for maximal binding in addition to optimising the reaction conditions was demonstrated.

The highest specific activity of CALA was measured when it was immobilised on magnetic beads with epoxy termination, even though the maximum amount of CALA was coupled to the beads with amino termination (Figure 17). The zeta-potential in buffer (pH 7) of the amino-terminated particles was positive (+0.6 mV), while that of the epoxy (-11 mV) or carboxy (-35 mV) terminated particles (Table 7) was negative. The zeta-potential in distilled water of the amino-terminated beads was positive (+18 mV), while that of the epoxy (-23 mV) and of the carboxy (-55 mV) terminated beads was negative (Table 7). The difference in the zeta-potential of the beads between using buffer or distilled water as medium of suspension reflects the influence of the sodium cations (M-PVA E02, M-PVA C12) and phosphate anions (M-PVA N12). The influence of the ion concentration on the electrophoretic mobility of particles was already investigated in several studies (Frimmel *et al.,* 2007 and Bolt *et al.,* 2005 and Kobayashi *et al.,* 2005). The polarity of the beads was reflected in their respective binding capacities with CALA, which has a negative zeta-potential at those immobilisation conditions (Table 7). With a decrease in the negative zeta-potential of the beads, the repulsion forces between particles and negatively charged CALA decreases which results in an increasing protein immobilisation. Accordingly, the amount of CALA coupled to the beads was maximum for the amino-terminated beads and was lower for the epoxy-terminated beads and least for carboxy-terminated beads (Figure 17). Nevertheless, the specific activity of CALA was the highest when it was immobilised on epoxy-terminated beads (M-PVA E02). This might be due to the hydrophobic nature of the epoxy beads, on which lipases have been shown to have higher specific activity (Carneiro-Da-Cunha *et al.,* 2002 and Lavayre *et al.,* 1982).

Previously, using the isoelectric focussing technique the isoelectric point (IEP) of CALA was determined to be 7.5 (Patkar *et al.,* 1993), which is significantly different

from what was observed in our studies using the zeta-potential measurements. The results show that the IEP for CALA is about 4 (Figure 18A). The difference in the IEP values is obviously caused by the different media conditions. The normal size of CALA remained relatively constant (10 – 100 nm) during the entire range of pH values studied, except at pH approximately 4, where the size increased to about 1000 nm. This is in agreement with the detection of IEP at pH 4, at which point the particles are known to agglomerate due to minimal repulsive forces.

The agglomeration of magnetic particles was observed near the IEP which shows the influence of the pH value. At the IEP (7.2) the strongest agglomeration of the M-PVA N12 beads was detected. At the lowest absolute value of the zeta-potential the electrostatic repulsion forces between magnetic particles are minimal, and they form large agglomerates with a particle size of about 30 µm (Figure 19).

Based on these results, it can be concluded that zeta-potential plays an important role in immobilisation of CALA to the activated magnetic beads. It can also be used as a predictor of particle agglomeration during the immobilisation process and also as a diagnostic tool to predict protein coupling to the particles.

7.4 Summary of the preparation and optimisation studies of immobilised *Candida antarctica*

The efficiency of binding during enzyme immobilisation depends not only on the chemical properties of enzyme and the matrix particle, but also on their surface potential. Zeta-potential quantifies the electrostatic interactions between enzyme and matrix particles, and can therefore, be used as an indicator of the binding efficiency in the enzyme immobilisation studies. In order to establish a correlation between the zeta-potential and the binding efficiency, we used CALA (*Candida antarctica* A-type Lipase) as a model protein for immobilisation on non-porous magnetic micro particles (1µm) with epoxy (M-PVA E02), carboxy (M-PVA C12) or amine (M-PVA N12) terminations. We observed maximal binding of CALA onto the M-PVA N12 beads, due to the electrostatic attraction between negatively charged proteins and carrier particles with neutral zeta potential. The binding of CALA was lower when M-PVA

E02 beads were used, followed by M-PVA C12 beads. The decreasing binding efficiency was obviously a result of the increasing electrostatic repulsion between the interaction partners. This was caused by increasing negative zeta-potential of the magnetic particles. Moreover, the medium of suspension of the particles also makes a significant difference. We found highest specific activity of the lipase immobilised on M-PVA E02 beads in buffer with medium concentration (0.3 M). These results demonstrate a clear correlation between zeta-potential and binding efficiency, advocating the possibility of using zeta-potential as a diagnostic tool in enzyme immobilisation.

8 Proof of principle: Integrated processing and multiple re-use of immobilised lipase by magnetic separation technology in mini pilot scale

Enzyme-catalysed chemical transformations are now widely recognised as practical alternatives to traditional (non-biological) organic synthesis, and as efficient solutions to synthetic processes (Koeller and Wong, 2001). Lipases are amongst the most important biocatalysts carrying out novel reactions in aqueous and non-aqueous media. This is primarily due to their ability to utilise a wide spectrum of substrates, to have high stability towards extremes of temperature, pH and organic solvents and to show chiral regio- and enantioselectivity (Koeller and Wong, 2001). In addition, there is great potential for using lipases to produce alkyl esters by direct conversion of triacylglycerols which can subsequently be used to synthesise value-added products, for instance biodiesel or biodegradable lubricants (ester oils) (Brenneis *et al.,* 2004). One option to render such processes more economic is the use of immobilised biocatalysts instead of free enzymes which cannot be recovered and re-used.

Recent work on magnetic separation of immobilised enzymes has been published in which the recovery and multiple re-use of the immobilised lipase was investigated in bench scale (Guo and Sun, 2004). Lipase from *Candida rugosa* was immobilised on hydrophobic and superparamagnetic microspheres and used as catalyst for esterification reactions. The enzyme was recovered by a bar magnet and exhibited good reusability in repeated batch reactions of 2 ml. In the solvent free system used, 73 % of the initial was found after 10 cycles (Guo and Sun, 2004). In another study a glucose oxidase-magnetite nanoparticle bioconjugate was used for glucose sensing (Rossi *et al.,* 2004). The glucose oxidase-coated magnetic particles remained active for two successive runs and thereafter lost activity. After five recycling runs 50 % of the activity remained. Authors speculated that the substantial loss of activity was probably due to loss of particles during the magnetic separation and re-dispersion cycles rather then a decrease in specific activity (Rossi *et al.,* 2004).

Hoffmann (2003) and Meyer (2004) have developed a fully automated high gradient magnetic separation system (HGMS) for the isolation of selected proteins from whey,

which could potentially be adapted to the separation and re-use of immobilised enzymes. The HGMS system contains a magnetic filter cassette (~ 46 ml volume) into which the particles are pumped and are captured. Non magnetic substances and the reaction mixture flow through the filter. The magnetic particles could then be washed or flushed out of the cassette by any buffer or liquid chosen in a process termed high gradient magnetic fishing (HGMF) (Hubbuch *et al.,* 2001, 2002 and Meyer, 2004). In the current work, adaptation of HGMS/HGMF was investigated for a mini-pilot semi continuous multicycle process for the re-use of *Candida antarctica* A-type lipase (CALA) immobilised on superpara magnetic micro particles.

8.1 Performance of the bench scale recycling studies of immobilised *Candida antarctica*

Immobilisation of the lipase: CALA was immobilised to M-PVA E02 as described in chapter 6.2 in this thesis. M-PVA E02 were resuspended in a volume of 0.1 M sodium phosphate buffer (pH 7) to give a concentration of 30 mg ml^{-1}. The lipase was then added, giving an enzyme concentration of 0.2 mg ml^{-1} and incubated with shaking overnight at room temperature. Immobilisation was stopped by magnetic capture of the particles and washing as described in chapter 6.2. The supernatant after immobilisation, as well as the washing fractions were collected and analysed for specific activity and protein content.

Two batches of 30 mg of immobilised enzyme were made exactly as described above and had an activity of 600 $U_{p\text{-NPP}}$ g^{-1} bead (batch 1) and 433 $U_{p\text{-NPP}}$ g^{-1} bead (batch 2) and were used in the bench scale and stability studies respectively. Subsequently the procedure was scaled up linearly to produce 5 x 600 mg batches which were combined (batch 3) giving an average activity of 420± 29 $U_{p\text{-NPP}}$ g^{-1} bead and were used for HGMS studies.

Study of enzyme stability: The free and immobilised CALA (batch 2) was resuspended in 0.1 M sodium phosphate buffer at different pH values (pH 6, 7, 8) and then incubated at different temperatures (4 °C and 25 °C). Samples were taken over a 38 day period and the specific activity of free and immobilised CALA was determined using a spectrophotometric assay with *p*-NPP as substrate.

Bench scale re-use studies with immobilised CALA: Small-scale studies were conducted in a temperature controlled and stirred reaction vessel with a final volume of 30 ml. The model reaction solution contained 29 ml of 10 mM sodium phosphate buffer (pH 8) with 1 ml tributyrin that had been previously homogenised with an overhead stirrer (Metrohm SM702) at medium speed (Figure 20). The reaction was started by introducing 20 mg of the immobilised enzyme with a bead related specific activity of 600 $U_{p\text{-NPP}}$ g^{-1} bead.

The reaction was followed by pH titration with 50 mM (NaOH) to hold the pH constant at 8.0 and the initial slope was used to calculate the reaction rate. After 50 min the immobilised enzyme was collected with a bar magnet (0.3 T) placed on the wall of the vessel and the reaction contents were collected for analysis. The immobilised enzyme was then washed 1-2 times with 10 mM phosphate buffer (pH 8) and centrifuged for 90 s at 10000 g in a bench top microfuge. The above sequence of events comprised one cycle and subsequently the washed immobilised enzyme was then added to fresh reaction solution in further cycles. Particle loss after each cycle was determined gravimetrically.

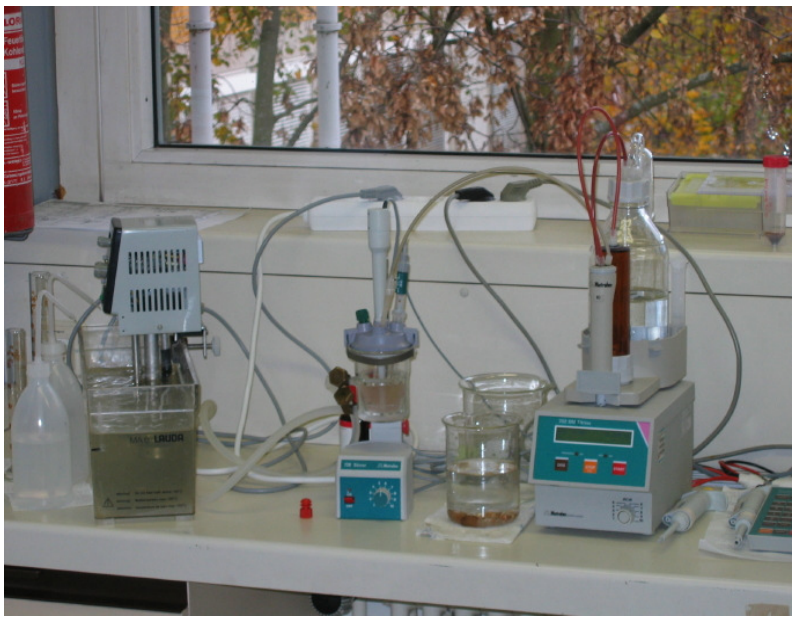

Figure 20: Titration equipment used for bench scale recycling studies of lipase of *Candida antarctica* A-type (CALA) .

8.2 Construction of the high gradient magnetic separator system for the recycling studies of immobilised lipase in mini pilot scale

Recovery and multiple re-use of immobilised CALA by HGMS: Mini pilot-scale studies were conducted in a temperature controlled (30 °C, 50 °C) and stirred reaction vessel with a final volume of 300 ml or 3000 ml. The model reaction solution contained 10 mM sodium phosphate buffer (pH 8) and 0.345 ml or 10 ml tributyrin that had previously been homogenised with an overhead stirrer at medium speed (Figure 21). The reaction was started by introducing up to 3 g of the immobilised lipase (batch 3) with a bead related specific activity of 420 $U_{p\text{-NPP}}$ g^{-1} bead.

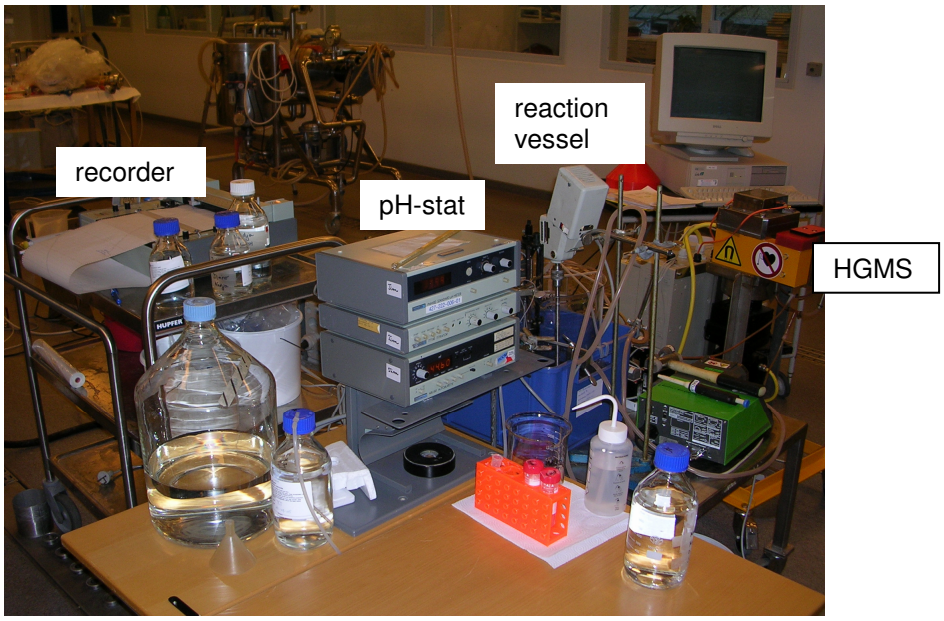

Figure 21: Integrated system for the multiple recovery and re-use of immobilised lipases of *Candida antarctica* A-type (CALA). The system is based on the high gradient magnetic separator equiped with a pH-Stat system and recorder for the detection of immobilised lipase activity.

The reaction was followe by pH titration with 50 mM NaOH, which kept the pH constant at 8, and the initial slope was used to calculate the reaction rate. Samples were taken for HPLC analysis and immediately frozen at -80 °C to avoid further hydrolysis of unreacted tributyrin to glycerol and butyrate. After the reaction had

proceeded for the desired time, the suspension was processed using the HGMS apparatus, the immobilised enzyme washed once with 10 mM phosphate buffer (pH 8), recovered and used for the next cycle. Particle loss after each cycle was determined gravimetrically. Four separate multicycle use and re-use campaigns were conducted under different conditions as mentioned in the results and discussion. After each campaign the immobilised enzyme was washed 5-7 times with 10 mM phosphate buffer (pH 7) and then used for the next campaign.

HGMS apparatus and system set-up: A laboratory type 'on-off' permanent magnet (Figure 22) based high gradient magnetic separator (Steinert HGF-10, Steinert Elektromagnetbau GmbH, Köln, Germany) was employed of the type designed for biological applications (Hoffman *et al.,* 2002).

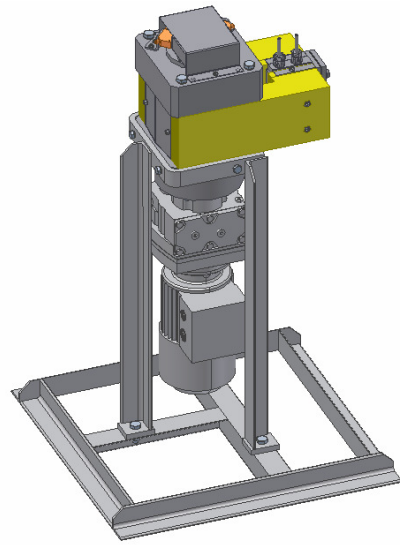

Figure 22: Laboratory type 'on-off' permanent magnet based high gradient magnetic separator (Steinert HGF-10, Steinert Elektromagnetbau GmbH, Köln, Germany) with magnetic filter cassette (in courtesy of Dr. Matthias Franzreb).

The separator had a 2.5 cm gap between the poles in which the magnetic filter was placed and a magnetic flux density of 0.32 T. A rectangular high-gradient magnetic filter (46 ml) as described by Ebner (2006) and Gomes (2006) was employed (Figure

23) and was packed with an alternating combination of 4 ferromagnetic mesh elements (DIN 1.4016, wire diameter of 0.315 mm; aperture of 1.00 mm) and 5 non-ferromagnetic mesh spacer elements (DIN 1.4301, wire diameter of 0.900 mm; aperture of 5.00 mm). In both cases, the mesh were plain woven cloths from Haver and Boecker, Oelde, Germany. The capacity of the filter cassette was known to be approximately 5 g of magnetic carriers when loaded at a superficial linear velocity of 20 m h^{-1} (Gomes, 2006) and preliminary experiments in this current work showed that 3 g of immobilised enzyme could be captured without breakthrough when pumping at 60 m h^{-1}. The amount of support loaded and the support amount released from the filter were determined by gravimetric analysis of the effluent.

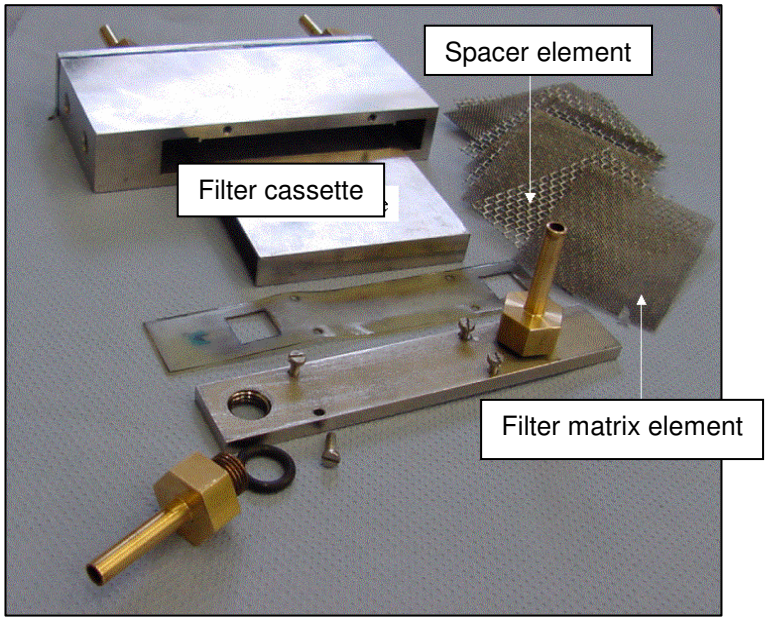

Figure 23: Construction of the magnetic filter cassette (in courtesy of Nikals Ebner).

The schematic of the setup designed here for immobilised enzymes is shown in figure 24 and consist of: a batch reactor (3000 ml or 300 ml) equipped with an overhead stirrer; a buffer container; an ethanol container, a collection beaker (3000 ml) and a single peristaltic pump (Masterflex Easy load model) connected together with 6 three-way solenoid switching valves (Burkert-Contromatic A/S, Herlev, Denmark) that were used to control the flow path direction. The HGF-10 magnet,

pump and valves were controlled by LabView software (Student Edition 6i, National Instruments Corporation, Austin, TX, 60.

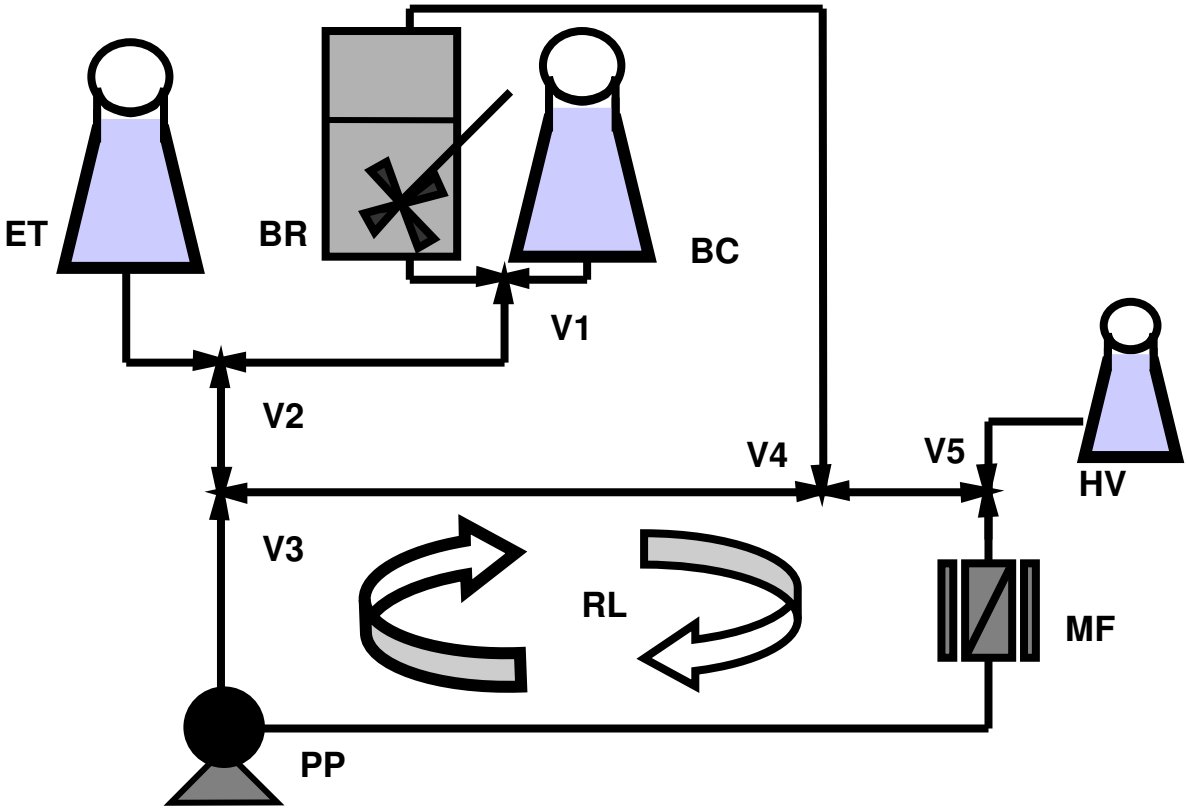

Figure 24: a) Schematic of the HGMS system used for multicycle processing with immobilised enzyme. MF = magnetic filter, PP = peristaltic pump, BC = buffer container; EC = ethanol container, RL = recycle loop, BR = batch reactor; HV = holding vessel, V1-5 = valves 1 to 5.

At the start of each campaign the reaction vessel (Figure 25) was filled with phosphate buffer, tributyrin added stirring initiated, and the system brought to the relevant operating temperature. The immobilised enzyme suspended in phosphate buffer was added and the reaction allowed to proceed for 20 min (at 30 °C) or 60 min (50 °C).

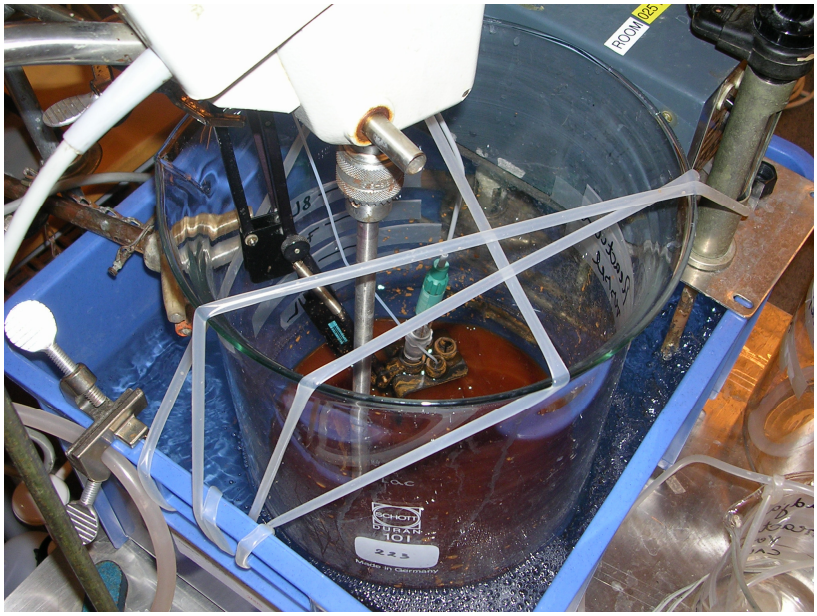

Figure 25: Reaction vessel with immobilised lipase on micro magnetic particles, overhead stirrer and probe.

Subsequently the reaction solution was fed at 60 m h^{-1} to the magnetic filter with the field on and the immobilised enzyme captured. The permeate was collected for later analysis. In no case was magnetic particle breakthrough observed. Subsequently washing buffer (10 mM phosphate pH 8) was fed into the system displacing all of the reaction solution in the lines and filter, the recycle loop was isolated by clothing valves V3, V4, V5 and the immobilised enzyme released by pumping at 100 m h^{-1} for 3 min in each direction with the field off. The particles were then flushed from the system into a holding vessel. The volume of the recycle loop and the filter was 200 ml. To maximise particles recovery, four further flushing cycles with mechanical vibration of the filter were conducted and the solution pooled, giving a combined volume of 1 L. Subsequently the system was flushed with 500 ml of 50 % ethanol to remove unreacted tributyrin and the flushings sent to waste. The reaction vessel was then refilled with phosphate buffer and tributyrin, the immobilised enzyme added without further washing and the above sequence of events were repeated until the campaign was completed.

Analytical methods: The activity of the immobilised enzyme on insoluble magnetic particles was measured using the spectrophotometric assay as described in chapter 6.2 in this thesis. In brief, the activity was measured directly at 410 nm in the reaction solution by using a modified *p*-nitrophenol ester assay (*p*-NPP) using a spectrophotometer without removal of the magnetic particles. The method was found to be effective for a wide range of magnetic bead concentrations from 0.01 to 0.2 mg ml^{-1}. One unit of activity ($U_{p\text{-NPP}}$) is defined as the cleavage of *p*-NPP at a rate of 1 µmol min^{-1} (chapter 6.2).

A pH stat system was used for the determination of the activity of free and immobilised lipase when reacting in tributyrin containing media. One unit of activity (U_{NaOH}) is defined as 1 ml 50 mM NaOH consumed per minute as measured from the initial slope of the titration curve in 10 mM phosphate buffer at pH 8 (chapter 6.2).

The tributyrin cleaving products were identified and analysed by HPLC using a Dionex system equipped with a RI and UV detector the latter at 210 nm. A Bio-Rad HPX87H column was used at 60°C employing an isocratic elution with 5 mM H_2SO_4 as the eluent. The retention time for glycerol was 13.25 min and the retention time for butyrate was 21 min.

The concentration of protein was determined using the Bradford assay (Bradford, 1976) using reagents from Biorad (Munich, Germany) and was conducted according to the manufacturer's specification.

The magnetic particle concentration was determined gravimetrically using a dry weight method as described in Heebøll-Nielsen (2002). Briefly, the dry-weight of the particles was calculated from the differences in mass before and after sample application and drying to pre-dried and weighed 47 mm diameter and 0.2 µm pore size filters (PALL Corporation, Ann Arbor, MI, USA).

8.3 Systematic studie on the new approach of multiple re-use of immobilised enzymes in mini pilot scale

The multiple recovery and re-use of immobilised CALA was attempted first in bench scale using a bar magnet for enzyme capture and then transferred to mini-pilot scale using an integrated HGMS system for enzyme handling. However, prior to recycling studies, the properties of the enzyme preparation were examined with the model oil substrate. Kinetic studies were conducted at 25 °C using tributyrin (34 g l^{-1}) and the same concentration of free or immobilised CALA (0.15 mg ml^{-1}). It was found that free CALA had a specific activity of 50 U mg^{-1} protein whilst the activity of the enzyme after immobilisation (batch 1) was reduced 4 fold to 11.25 U mg^{-1} protein. This corresponded to an immobilised enzyme activity of 600 $U_{p\text{-}NPP}$ g^{-1} magnetic beads. The stability of the immobilised enzyme (batch 2) at pH 7 was also examined. The results in figure 26 showed that at 4°C and pH 7 the immobilised lipase retained 90 % of its initial activity after 38 days and had stability which was comparable to, or better than, the free enzyme. At 25 °C at pH 7 the immobilised enzyme lost 34 % of the initial activity over 38 days, whereas the free enzyme lost only approximately 20% activity over a similar period. The reasons for this are not clear, but must be due in some way to immobilisation on the matrix. Buchholz *et al.* (2005) state that in the electrical double layer formed by charges on the surface of particles with immobilised enzymes, very high local pH and conductivity values can occur. These high values could be expected to accelerate the loss of activity of the immobilised enzyme seen in the current work. Furthermore, it is known that immobilisation can change the intrinsic properties of an enzyme (Buchholz *et al.,* 2005). In the current work the immobilised enzyme had 4 times lower activity than the free CALA (i.e. based on the amount of protein), it can thus be speculated that distortion of the enzyme during inactivation could make it more susceptible to deactivation by high local pH values. Alternatively, slow further reaction of the immobilised enzyme with unreacted epoxy groups on the surface might restrict the enzymes active site or conformational changes. Further work is required to improve the stability and activity of the immobilised enzyme.

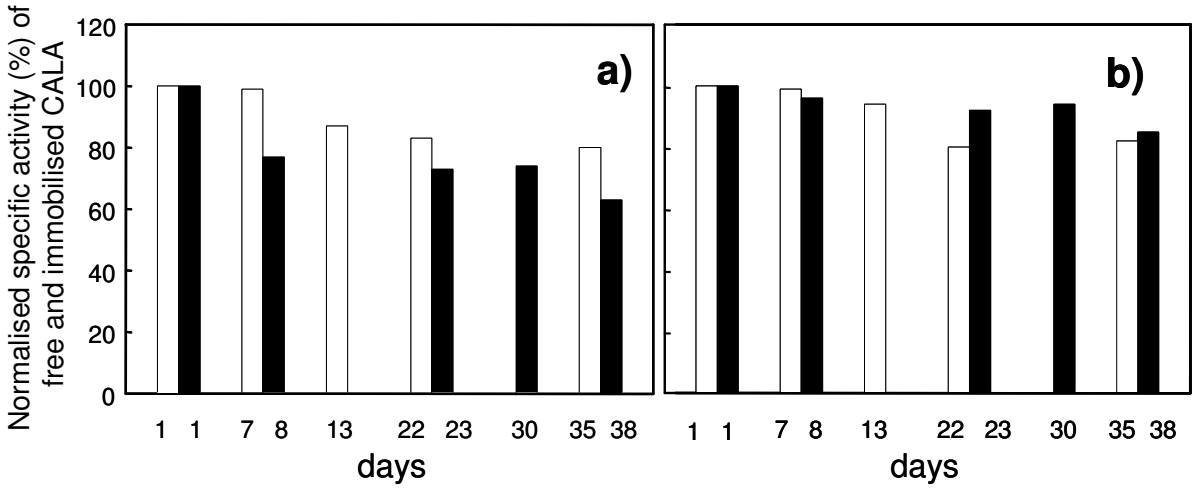

Figure 26: Stability of free CALA (□) and immobilised CALA (■) at 25 °C (a) and 4 °C (b) in 0.1 M sodium phosphate buffer at pH 7. 100 % of activity of the immobilised CALA corresponds to 433 $U_{p\text{-NPP}}$ g^{-1} bead. 100 % activity of the free CALA corresponds to 84 $U_{p\text{-NPP}}$ mg^{-1} protein. In all cases the specific activities were measured using the substrate *p*-NPP in a spectrophotometric assay.

8.3.1 Bench scale batch studies of the recovery and multiple re-use of immobilised CALA

In total, 8 recovery cycles of immobilised CALA (batch 1) in bench scale were performed at 30 °C with 34 mg ml^{-1} tributyrin. In each cycle the specific activity was determined from the initial rate of NaOH consumption during tributyrin hydrolysis and the starting specific activity of the immobilised CALA was 300 U g^{-1} bead under these conditions. Multiple recovery and re-use was possible 5 times without any loss of activity. However after 8 recovery cycles 73 % of the initial specific activity of the immobilised CALA remained. In addition, 22 % of the particles were lost after 8 cycles. Thus the average consumption of the added 34 mg ml^{-1} tributyrin was 6 % per cycle (50 min) in the first cycle and fell to 4.8 % in the eigth cycle.

8.3.2 Multiple recovery and re-use of immobilised CALA with HGMS in mini pilot scale

The bench scale experiments indicated that the enzyme was stable for 5 cycles at 30 °C and thus a multicycle campaign using HGMS system was conducted. The results in figure 27 show eight repeated cycles comprising campaign 1 and in each, 3000 ml

of 0.12 mg ml^{-1} tributyrin was treated at 30 °C. Subsequently the temperature was raised and 4 further cycles were conducted at 50 °C or 40 °C (campaign 2). In all cycles the amount of magnetic particles used remained essentially constant at 3 ± 0.43 g. The results for campaign 1 show that the total amount of NaOH consumed over the 1 h reaction time in the first cycle was ~47 ml, corresponding to ~100% consumption of tributyrin. The amount of butyrate formed was found by HPLC to be 0.05 mg ml^{-1} corresponding closely to that expected (0.06 mg ml^{-1}). The 7 subsequent cycles in campaign 1 showed a steady decrease in the amount of tributyrin converted to 77 % of that observed in cycle 1. Likewise the initial activity of the immobilised enzyme measured from the initial slope of the NaOH consumption profile dropped to 75 % from cycle 1 to cycle 8. It should be noted that the immobilised enzyme was only washed once between each cycle and it was suspected that the loss of activity observed was caused by lack of washing. This was confirmed by using a more rigorous washing procedure after cycle 8 (consisting of 1 wash in phosphate buffer then 2 washes in water and finally 2 washes in phosphate buffer) which yielded an activity of 500 $U_{p\text{-NPP}}$ g^{-1} (using the spectrophotometric assay) compared to an initial activity prior to cycle 1 of 420 $U_{p\text{-NPP}}$ g^{-1} (Table 9).

Table 9: Summary of the conditions used in each campaign and conversion of tributyrate to butyrate.

Campaign	Temp. °C	Total volume treated (l)	Tributyrin [mg ml^{-1}]	Bead related spec. activity* [U g $particle^{-1}$]	Average butyrate concetration detected ** [mg ml^{-1}]
0	na	na	na	500	na
1	30	2	0.12	550	0.05 (0.06)
2	50	12	0.12	450	0.06 (0.06)
3	30	1.2	34	-	2.09 (1.53)
4	50	1.2	34	60	2.15 (1.19)

na: not applicable – : not analysed

* measured with spectrophotometric assay using *p*-NPP as substrate.

** number in paranthesis are the theoretical expected concentration based on the amount of NaOH consumed during each compaign.

In light of the successful regeneration of the immobilised enzyme, the catalyst was thus applied to a further 4 cycles in campaign 2 (figure 27) under the same conditions as campaign 1, but at 50 °C, a temperature closer to the optimum for CALA of 70 °C (Kirk and Christinsen, 2002).

The activity in cycle 9 measured by NaOH consumption was thus very much higher than in campaign 1 and the total amount of tributyrin converted was essentially the same as in cycle 1 (i.e. corresponding to total tributyrin conversion). The drop in initial activity and in NaOH consumed was also found to be a consequence of not washing the enzyme between cycles as ~90 % of the activity prior to cycle 1 could be measured using the spectrophotometric assay after cycle 12 following the washing protocol employed at the end of campaign 1 (Table 9). The effects of a lowered temperature (40 °C in cycle 11 only) are clearly apparent (figure 27).

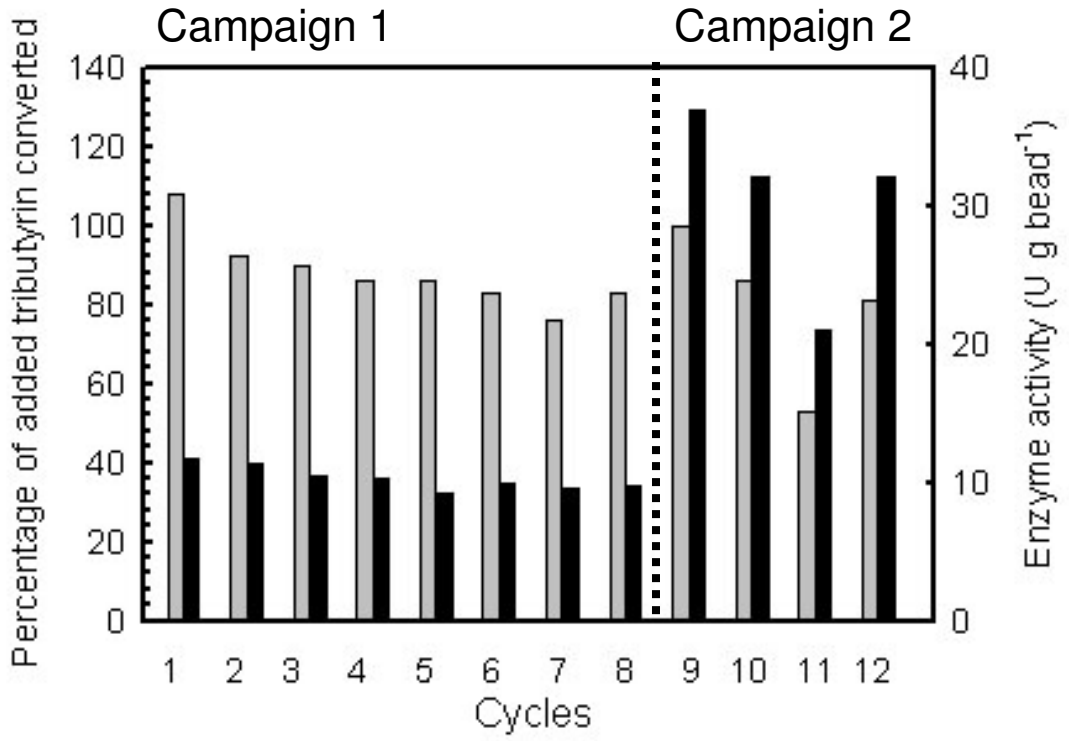

Figure 27: Multiple recovery and re-use of immobilised CALA with HGMS in mini pilot scale. Campaign 1 (cycle 1-8) and 2 (cycle 9-12) were conducted at 30 °C and 50 °C, respectively (exept for cycle 11 which was at 40 °C) with 3000 ml of fresh 0.12 g l^{-1} tributyrin in each cycle. The enzyme activity in U g $bead^{-1}$ (■) is defined as µmol butyrate liberated per minute and is calculated from the amount of 50 mM NaOH titrated to neutralise the butyrate formed during the reaction in 10 mM phosphate buffer at pH 8. The percentage of tributyrin converted (□) over 60 min or 20 min of reaction was calculated from the total volume of NaOH added in campaign 1 and 2, respectively.

8.3.3 Multiple recovery and re-use of immobilised CALA with HGMS in mini pilot scale from a reaction media containing 35 g l^{-1} of tributyrin

Following the success at campaign 1 and 2 and the high residual activity of the immobilised enzyme, 1.5 g of the preparation was re-used in two further campaigns with 290 fold higher tributyrin concentrations (Table 9). The results in figure 28 (for campaign 3 (30 °C) and campaign 4 (50 °C)) show similar trends as were seen in campaign 1 and 2, with a steady decrease in tributyrin conversion as more cycles were completed and the highest enzyme activity and substrate conversion observed at 50 °C. However, the much higher tributyrin concentration employed resulted in 5 to 10 fold higher apparent activity of the enzyme (measured by NaOH consumption) then seen in campaigns 1 and 2. In cycle 13, 9 % of the tributyrin offered was degraded in the 20 min reaction time employed and 2 mg ml^{-1} butyrate was measured by HPLC. Campaign 4 (at 50 °C) followed directly on from campaign 3 after washing and regenerating the enzyme. However, following the 20th cycle, washing and regenerating of the immobilised enzyme resulted in only 14 % of the activity prior to campaign 1 when measured with the spectrophotometric assay (Table 9).

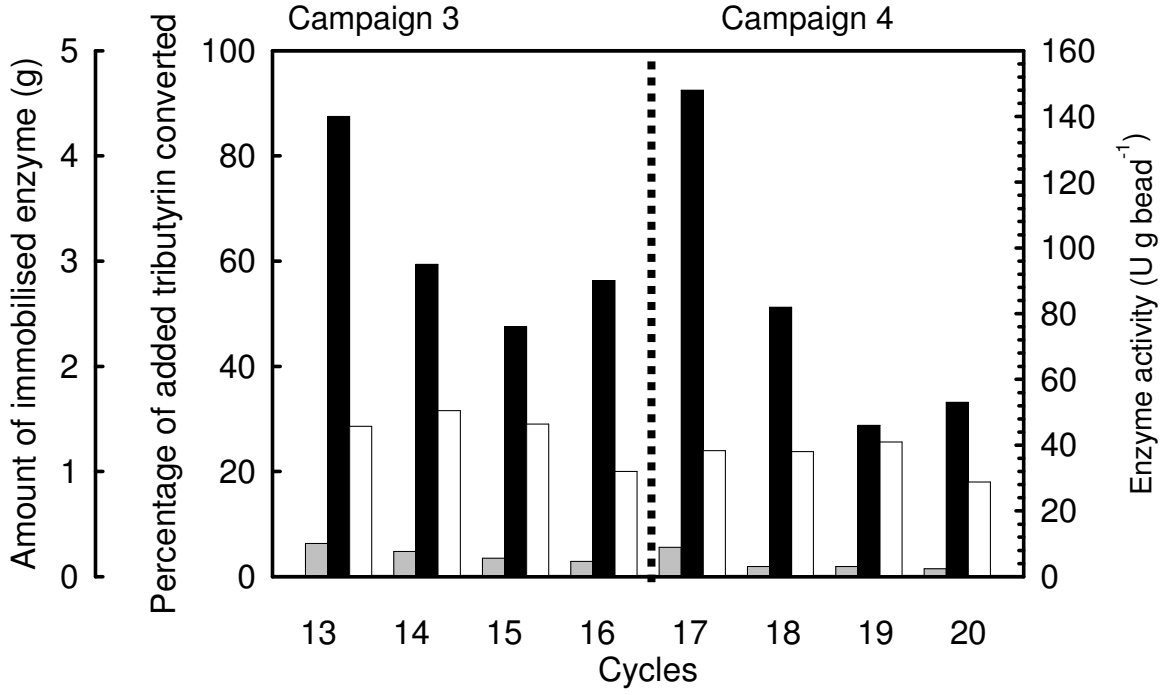

Figure 28: HGMS in mini pilot scale for the repeated treatment of 300 ml and 34 g l^{-1} tributyrin. Campaign 3 (cycles 13-16) and 4 (cycle 17-20) were conducted at 30 °C and 50 °C respectively. Enzyme activity (■) and the percentage of tributyrin converted (□) were determined as in figure 27. Amount of immobilised enzyme (□) recovered and re-used in each cycle.

8.4 Potential for integrated processing and multiple reusability of immobilised *Candida antarctica* by magnetic separation technology

The results presented here showed a decline in apparent activity of the immobilised enzyme from cycle to cycle during HGMS processing, which could be reversed by employing a thorough washing protocol until the enzyme had been re-used 20 times. Subsequently regeneration was not possible using the protocols employed here. Loss of immobilised enzyme activity during cycles of reuse has been observed by other workers and various causes have been proposed. For example Rossi *et al.* (2004) claimed that mechanical abuse during separation may lead to activity loss, although no direct evidence for this was seen in the current work. Those authors also found that a decrease in the total activity in their reaction was due to loss of particles during the separation process employing a bar magnet (Rossi *et al.,* 2004). Such losses were not seen from cycle to cycle in the current work due to the efficient trapping of the immobilised enzyme using HGMS even in the most challenging solutions employing 34 mg ml^{-1} tributyrin, which were viscous and caused apparent aggregation of the catalyst. However, ensuring complete release of the immobilised enzyme from the HGMS filter was of greater concern, particularly using the media containing 34 mg ml^{-1} tributyrin and an extended period of recirculation and filter vibration was required. Similar difficulties in release of magnetic adsorbents during HGMF processing have also been reported (Hubbuch *et al.,* 2001; Ebner, 2006; Gomes, 2006; Franzreb *et al.,* 2006) and indicate a need for development of improved HGMS separators for biological systems. Nevertheless in the current work loss of immobilised enzyme particles from cycle to cycle was of minor concern (see e.g. Figure 28).

Rossi *et al.* (2004) proposed that aggregation of magnetic particles could result in partial loss of immobilised enzyme activity in their bench scale studies due to reduced accessibility of the substrate molecules to the enzyme. As noted above, the magnetic particle immobilised enzyme studied here showed a strong tendency to agglomerate in the oil-water emulsion formed in the system containing 34 mg ml^{-1} tributyrin. A slimy type of fluid was formed rather than a suspension, particularly in campaign 4 in which incubations at 50 °C were used. Such aggregation was present, although much less evident when using only 0.12 mg ml^{-1} tributyrin. Thus

aggregation in the oil-water emulsion employed here appears to be the main contributor to the decline in cycle to cycle activity seen, which could be reduced by extended washing. However, an irreversible denaturation of the enzyme is believed to have occurred during campaign 4, which may be related to elevated temperatures employed there. Although CALA is more thermostable than many enzymes, accelerated activity loss is to be expected as temperature is raised (see e.g. figure 26). Nevertheless, further work on optimisation of washing protocols seems warranted in order to maintain the best enzyme activity in a process, without excessively to costs and generation of large volume of waste.

One generic advantage of HGMS is that very challenging crude feedstocks can be treated due to the high voidage of the filters and the technique has thus been applied successfully since the 1970's for mineral processing and waste water treatment (Svoboda, 1987). Furthermore, several examples of the successful affinity purification of enzymes in mini pilot scale (e.g. superoxide dismutase and lactoferrin) from whey have recently been demonstrated (Hoffmann, 2003; Meyer *et al.,* 2005; Franzreb *et al.,* 2006). Unwanted non-magnetic contaminants can simply pass through or be washed out without blocking the HGMS filter. Given the proceeding reports and in light of the work conducted in the present study, it is concluded that HGMS shows great potential for biocatalytical applications necessitating the recovery and re-use of immobilised lipases or other enzymes form crude, oily and challenging feedstocks.

8.5 Summary of the proof of principle of multiple recovery and re-use of immobilised lipase by magnetic separation technology

High-gradient magnetic separation based processing is demonstrated for the semi-continuous multicycle re-use of a lipase immobilised on magnetic micro particles. The lipase of *Candida antarctica* A-type (CALA) was immobilised on polyvinyl alcohol coated magnetic particles (1-2 µm diameter) with epoxy functionalisation. The immobilised CALA was used to hydrolyse a model oil-water 2-phase-system composed of a phosphate buffer with tributyrin at up to 3 litre scale. The immobilised enzyme was subsequently recovered in a magnetic filter using high-gradient magnetic separation and reapplied in repeated cycles of hydrolysis and recovery. Two different temperatures of 30 °C and 50 °C, tributyrin concentrations (0.12 g l^{-1}

and 35 g l^{-1}) and reaction times were tested. In each case the reaction was followed by pH titration using NaOH, as well as by HPLC analysis. Consecutive cycles were conducted for each reaction condition and in total the immobilised CALA was subjected to 20 recovery and re-use cycles, after which ~14 % of the initial specific activity still remained.

9 Conclusion and outlook

After intensive investigation and development it was demonstrated for the first time; the semi continuous multicycle re-use of lipase immobilised on magnetic micro particles (Bio-Polymer Technology AG, Baesweiler, Germany) with the help of high gradient magnetic separation technology (HGMS) in mini pilot scale. The model system for the enzymatic conversions was mainly represented by the commercial lipase of *Candida antarctica* A-type (CALA) working in a 2-phase-system of tributyrin and phosphate buffer. The feasibility of the biocatalytic conversion of hard to solve substrates or substrates in emulsions (tributyrin/buffer) with CALA immobilised on poly vinyl alcohol (PVA) magnetic micro particles (1µm) was shown. A basic high gradient magnetic separator (HGMS) suitable for the needs of the biological sector and developed by the research center of Karlsruhe was employed.

Basic studies for the development of a suitable spectrophotometric assay for online measurement of the activity of lipase immobilised on micro magnetic particles were necessary. Thus, it was possible to screen different lipases immobilised on various magnetic particles for the desired properties.

Subsequently, the optimisation of lipase immobilised on micro magnetic particles with three different terminations (amino-, epoxy or carboxy groups) were investigated by zeta-potential measurement as a diagnostic tool. It was found that epoxy activation of PVA coated magnetic particles prior to immobilisation of CALA gave the best preparation. The storing stability of free and immobilised CALA was investigated at different pH values and temperatures and optimal conditions were defined.

The last part of this thesis combined the techniques and the knowledge gained earlier. The proof of principle of integrated processing and multiple re-use of immobilised lipase by magnetic separation became possible at different temperatures and tributyrin concentrations. Immobilised CALA was used to degrade a model oil-water 2-phase-system (tributyrin/buffer), then recovered in a magnetic filter using HGMS and then applied in up to 20 new cycles.

The investigation steps successfully achieved on their way to realise the multiple recovery and re-use of immobilised lipases in mini pilot scale are outlined in figure 29.

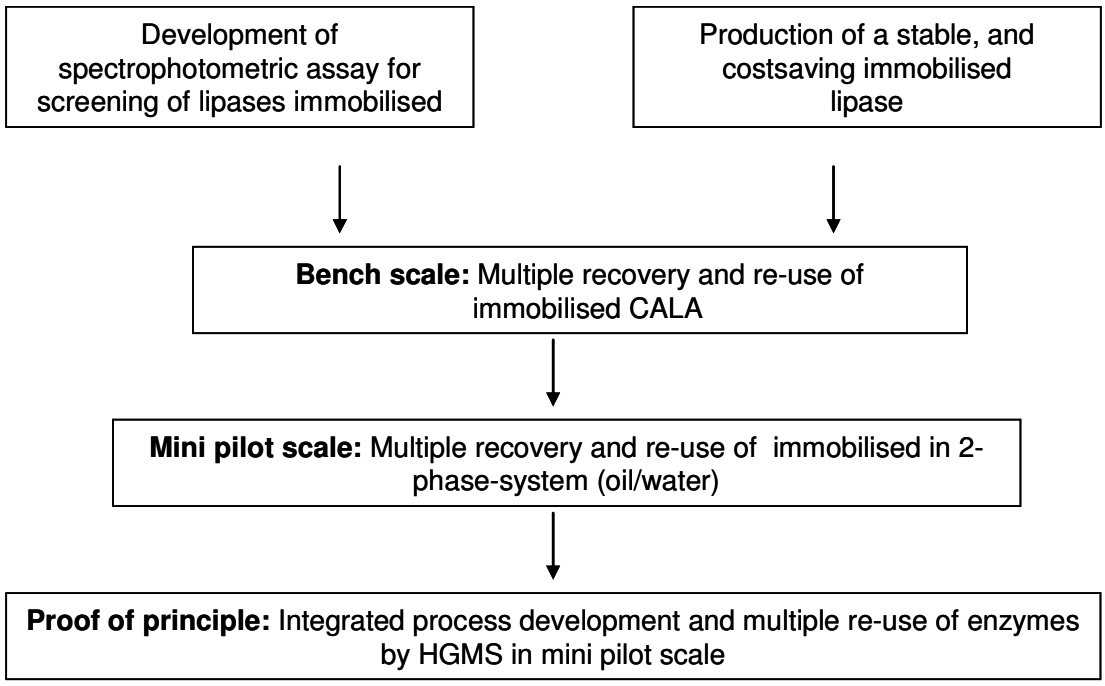

Figure 29: Main points achieved on the way to realise the multiple re-use of immobilised lipase by HGMS in mini pilot scale.

From this work it can be concluded that the spectrophotometric assay developed here can be expected to be useful for fast screening of immobilisation chemistries for other enzymes and could probably be implemented in 96 well microtiter plate format and with robotic spectrophotometers. The rapidness of the assay, and reduction in handling steps (i.e. no removal of immobilised enzyme or stirring required during measurement) may provide an opportunity for complete automisation of the method, e.g. via flow injection analysis to permit online measuring of activity in HGMS based industrial processes, which will require monitoring of the enzyme activity during the process and before re-use.

Based on the results of the optimisation studies of lipase immobilisation, it can be concluded that zeta-potential plays an important role in immobilisation of CALA onto the activated magnetic beads. It can also be used as a predictor of particle

agglomeration during the immobilisation process and also as a diagnostic tool to predict protein coupling to the particles.

The proof of principle that HGMS based processing is possible for the semi-continuous multicycle re-use of CALA immobilised on micro magnetic particles was demonstrated. HGMS provides an alternative method for multiple recovery and re-use of immobilised lipases. The results presented here are promising for the extended use of immobilised enzymes not only in 2-phase-systems for hydrolysis of oils but also in other enzymatic systems like two-step one vessel reaction systems or for the use of immobilised proteases.

The loss of activity of immobilised CALA during the separation campaigns needs to be discussed but has been observed by other authors as well that had been working on bench scale magnetic separation. Rossi *et al* (2004) claim to observe mechanical abuse by magnetic separation or by aggregation of the magnetic particles which could result in partial loss of the enzymatic activity due to limited accessibility of the substrate molecules to the enzyme. Another reason for the decrease of the total activity is the loss of particles during the separation process (Rossi *et al.,* 2004) especially when the separation is performed with a bar magnet. The loss of particles could be improved in the studies here by using HGMS with a capable on/off magnet of about ~0.32 T. Furthermore it was observed that the activity of the immobilised CALA could be regenerated (campaign 1-4) by washing the particles with distilled water and phosphate buffer. The recovery of the particle related specific activity could be proved by measurement with the spectrophotometric assay and the ph stat assay. However, there was a strong decrease of particle related specific activity during the last campaign (4). A possible reason for that is that the particles showed a strong tendency to agglomerate in the oil/water system containing 34 mg ml^{-1} tributyrin. Furthermore the separation of the viscous media especially at 50 °C caused problems as it became difficult to separate with the cassette of the HGMS properly.

Advantageous with magnetic separation is that immobilised enzymes can be separated easily from viscous and oily media or 2-phase-systems and batch

adsorption can tolerate particle loaded media. Furthermore, unwanted contaminants can simply be washed away without blocking the column, the filters or the separation device (Hohlschuh, 2005). Several examples for the successful affinity purification of enzymes in mini pilot scale (e.g. superoxid dismutase and lactoferrin) from whey have recently been demonstrated (Meyer, 2004 and Hoffmann, 2003). Furthermore the HGMS system originally was developed for the water purification in the metal industry and has proven to be suitable in the application on nasty feedstocks. Thus the HGMS system possibly bears potential for biocatalytical applications such as the recovery and re-use of immobilised lipases form crud, oily and challenging feedstocks.

In future works, it would be pathbreaking for enzyme immobilisation, to develop specified tools to realise the directed binding of enzymes to matrix particles. Enzymes could be “forced” to get immobilised in their most active conformation. Thus, most likely a computer based modelling tool should be able to predict the surface potential of different areas of the matrix body at different pH values and molarities of buffers.

Furthermore, the use of immobilised enzymes especially in 2-phase-systems could be improved by the use of “smart magnetic particles” with long spacer arms. Especially lipases that have a lid are active only at the interface of the oil/water emulsion. Long spacer arms for the immobilisation of lipases might allow easier access to the substrate, as the enzyme would “stick out” in the media. Lipases that are fixed close to the matrix might undergo diffusional limitations while accessing the substrate.

A following project based on the success of the multiple re-use of immobilised CALA by HGMS would be the multiple re-use of immobilised cellulases. Cellulases are used for the degradation of lignocellulosic raw materials (often waste materials from agriculture and industry) consisting of hexoses and pentose sugars to glucose. Thus cheap and sustainable lignocellulosic raw materials can be recycled for the fermentation of yeast for ethanol production.

Another actual research project profiting from the successful proof of principle of the multiple re-use of immobilised enzymes by HGMS is based on the use of immobilised trypsin. Trypsin immobilised on magnetic micro particles is used here for the fishing of a trypsin (Bowman-Birk Inhibitor (BBI)) inhibitor from industrial crude soy liquids. Soy liquids are valuable sustainable raw materials and especially BBI can be used in medial applications such as cancer treatment.

10 Appendices

10.1 Literature

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10.2 Symbols and abreviations

Latin symbols

a	year	
m	meters	
B	magnetic flux density	T
F	force	N
H	magnetic field strength	H
H_c	magnetic coercivity	A m^{-1}
L	length	m
M	magnetisation	A m^2 kg^{-1}, A m^{-1}
V_p	particle volume	m^3

Greec symbols

μ	magnetic permeability
μ_o	permeability constant of vacuum
χ	magnetic susceptibility
ε_{410}	extinction coefficient at $_{A410}$

Abreviations

BSA	bovinie serum albumine
CALA	lipase of *Candida antarctica* A-Type (CALA)
CALB	lipase of *Candida antarctica* B-Type (CALB)
FFA	free fatty acid
BioMag	micro sized silane coated magnetic carrier (Polyscience, UK)
HGMF	high gradient magnetic fishing
HGMS	high gradient magnetic separation
M-PVA	poly (vinyl alcohol) magnetic beads
NHS	n-hydroxy succinimide
TAG	triglyceride
DAG	diglyceride
p-NPP	para-nitrophenylpalmitate
p-NP	para-nitrophenol