

Thesis for the degree of Doctor of Philosophy

**Immobilization of feruloyl esterases in  
mesoporous silica**

Linking immobilization conditions, microenvironment  
and material properties to enzyme activity

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Department of Chemical and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2014

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Cover: Illustration of a feruloyl esterase entering a mesoporous particle.

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## **Immobilization of feruloyl esterases in mesoporous silica**

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### **Abstract**

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Feruloyl esterases are a class of enzymes that can be used as biocatalysts for the modification of bioactive hydroxycinnamic acids. In industry, enzyme immobilization is often necessary to stabilize the enzymes and to allow their reuse. Mesoporous materials have become a popular immobilization support due to advantages such as large surface area, high mechanical stability and the possibility of adjusting the pore size to the dimensions of the enzyme. The hypothesis investigated in this work was that mesoporous material would provide a robust immobilization support for feruloyl esterases, and would not suffer from the problem of enzyme inactivation reported in previous studies. As well as excellent reusability and high yields in the transesterification of methyl ferulate to butyl ferulate, the feruloyl esterases immobilized in mesoporous silica exhibited altered product selectivity. This indicated the need for deeper studies into how enzymes are affected by the immobilization process, and which factors and material properties influence enzyme activity. A novel method for real-time studies of the immobilization process was therefore developed, based on quartz crystal microbalance with dissipation monitoring. The immobilization process was found to occur in two stages, with initial covering of the outer surface followed by filling of the pores. Proof that the enzymes indeed enter the pores was also found, which is important as it is usually assumed that the enzymes are located inside the pores. Furthermore, modeling of the enzyme structure gave new insights on the structure-activity relationship as a function of pH, and was linked to an apparent structural memory effect from the adsorption to the mesoporous material. Differences in the microenvironment inside the pores were characterized by developing a new method for measuring the local pH through fluorescent labeling of the enzymes, which showed a buffering effect towards neutral pH inside the pores. The aim of the present work was to link the new knowledge obtained on the microenvironment, immobilization conditions and material properties of the mesoporous material to their effects on enzyme activity. The use of immobilization as a means of controlling enzyme activity is important for the development of more rationally designed biocatalysts based on enzymes immobilized in mesoporous materials.

**Keywords:** Enzyme immobilization, Biocatalysis, Mesoporous materials, Silica, Feruloyl esterases, Hydroxycinnamic acids, QCM-D, Protein modeling.

## List of publications

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This thesis is based on the work described in the following papers:

- Paper I: **Thörn C**, Gustafsson H, Olsson L. *Immobilization of feruloyl esterases in mesoporous materials leads to improved transesterification yield.* J Mol Catal B: Enzym. 2011; 72:57-64.
- Paper II: **Thörn C\***, Gustafsson H\*, Olsson L. *QCM-D as a method for monitoring enzyme immobilization in mesoporous silica particles.* Microporous Mesoporous Mater. 2013; 176:71-77.
- Paper III: **Thörn C\***, Udatha DBRKG\*, Zhou H, Christakopoulos P, Topakas E, Olsson L. *Understanding the pH-dependent immobilization efficacy of feruloyl esterase-C on mesoporous silica and its structure-activity changes.* J Mol Catal B: Enzym. 2013; 93:65-72.
- Paper IV: **Thörn C**, Carlsson N, Gustafsson H, Holmberg K, Åkerman B, Olsson L. *A method to measure pH inside mesoporous particles using protein-bound SNARF1 fluorescent probe.* Microporous Mesoporous Mater. 2013;165:240-246.
- Paper V: Carlsson N\*, Gustafsson H\*, **Thörn C\***, Olsson L, Holmberg K, Åkerman B. *Enzymes immobilized in mesoporous silica: a physical-chemical perspective.* Adv. Colloid Interface Sci. 2014; 205:339-360.

\* These authors contributed equally to the study.

Published paper not included in this thesis

Gustafsson H, **Thörn C**, Holmberg K. *A comparison of lipase and trypsin encapsulated in mesoporous materials with varying pore sizes and pH conditions.* Colloids Surf, B. 2011;87:464-71.

## The author's contributions

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- Paper I: I designed and carried out all the experimental work, apart from the synthesis of the mesoporous materials. I analyzed the results and wrote the manuscript.
- Paper II: I, together with Hanna Gustafsson, developed the idea, designed and carried out the experimental work, analyzed the data, and wrote the manuscript.
- Paper III: I designed the study, carried out the experimental work and analyzed the results. I was involved in planning the modeling and the analysis of the results, though Gupta Udatha performed all the modeling and docking studies. I wrote half the manuscript.
- Paper IV: I conceived the idea of probing the enzyme. I designed and carried out the experimental work together with Nils Carlsson. I wrote half the manuscript.
- Paper V: I, together with Hanna Gustafsson and Nils Carlsson, contributed equally in writing this review.

## Preface

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The work described in this thesis was performed from within the Linnaeus Centre for Bio-inspired Supramolecular Function and Design (SUPRA), which is funded by the Swedish Research Council (Vetenskapsrådet; project 349-2007-8680). We formed an “enzyme cluster” within the framework of SUPRA, which intended to bring together expertise from different groups and using the interdisciplinary collaboration to allow new approaches to research questions within enzyme immobilization and interaction between molecules. Hanna Gustafsson and Krister Holmberg from the group of Applied Surface Chemistry came into the project with expertise on synthesis and development of different types of mesoporous materials. Nils Carlsson and Björn Åkerman from the group of Physical Chemistry focused on using spectroscopic methods for characterization of the immobilized enzymes. I and Lisbeth Olsson from the group of Industrial Biotechnology had the biological perspective with focus on the application of enzymes for biocatalysis purposes.

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# 1 Introduction

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All biological life depends on chemical reactions catalyzed by enzymes. Therefore, it is not surprising that industry utilizes these natural catalysts in a broad range of applications. Most of the enzymes produced are used either in household products such as detergents, or in the food and beverage processing industry to change properties such as flavor and nutritional value. Enzymes are also key components in the production of biofuels such as ethanol and diesel.

Inorganic compounds with catalytic capacity have historically been dominant in classical chemical synthesis requiring catalysts, however, these processes are often not sustainable from an environmental point of view. If the same reactions could instead be catalyzed by enzymes, this would allow for more sustainable processes. The fatty acids aspartame and acrylamide are two examples of bulk chemicals that can now be produced through biocatalysis [1]. Enzymatically catalyzed reactions are normally run under milder conditions (pH often close to neutral and physiological temperatures), which should reduce the amount of hazardous waste, lower the energy consumption and lead to overall safer processes [2]. Furthermore, enzymes are generally regio- and enantioselective, which means that the enzyme can modify a certain part of a complex molecule, while at the same time allowing control of its chirality and stereochemistry.

However, replacing an inorganic catalyst that has been used in the synthesis of a bulk chemical for decades with an enzymatic catalyst is not a simple task. Many applications employing enzymes, developed for industrial use during the past decade, have been directed towards the production of compounds used in pharmaceuticals [3]. This is where the advantage of enzymes being able to perform selective reactions on complex molecules becomes most evident. The scope for cost reduction is also greater for complex molecules that generally require multistep processes when synthesized with classical chemical catalysis. One of the most famous examples is the synthesis of atorvastatin (Lipitor), where many of the intermediate synthesis steps can now be catalyzed with enzymes (e.g. ketoreductases and aldolases) [4]. Hydrolases are another class of enzymes that have attracted attention due to their ability to cleave carbon-oxygen bonds. The lipase *Candida antarctica* lipase B is among the most frequently used enzymes in biocatalysis, as it has a broad substrate specificity that allows esterification of a wide range of compounds [5]. Feruloyl esterases, studied in the

present work, can sometimes be classified as lipases, and generally have a similar catalytic triad. The reason for using feruloyl esterases is that, due to their specificity, they are suitable for the modification of the antioxidant compounds called hydroxycinnamic acids. More information on the bioactivity of hydroxycinnamic acids and the catalytic activity of feruloyl esterases is given in Chapter 2.

Most enzymes used in chemical synthesis have been engineered to some extent to achieve satisfactory activity, stability and selectivity [3, 6]. The structure of the enzyme is usually altered through the deletion, replacement or addition of amino acids. The main challenge in enzyme engineering is the lack of knowledge on how molecular interactions affect the structure and activity of enzymes [3, 7]. This means that the rational design of new enzymes, with novel activities, and the prediction of their activities and selectivity are still limited, and a second round of structural optimization of the enzyme activity is often necessary. However, the area of protein engineering is evolving rapidly thanks to the development of many bioinformatics tools, which have made virtual screening of enzyme structure-activity relationships more reliable [6].

An active enzyme is generally not sufficient to ensure a sustainable and economically feasible biocatalytic process. Immobilization of the enzyme can facilitate enzyme reusability and product purification and, in some cases, further improve enzyme activity and stability. The process of developing a biocatalyst is illustrated in Figure 1, where the different topics of the papers included in this thesis, and their relation to the design of a biocatalyst, are also indicated. The iterative design process involves a typical trial-and-error approach. To allow a more rational design of the immobilization process, and of the support material itself, it is important that we have a better understanding of how the activity and function of the enzyme are affected by its interactions with the support material.

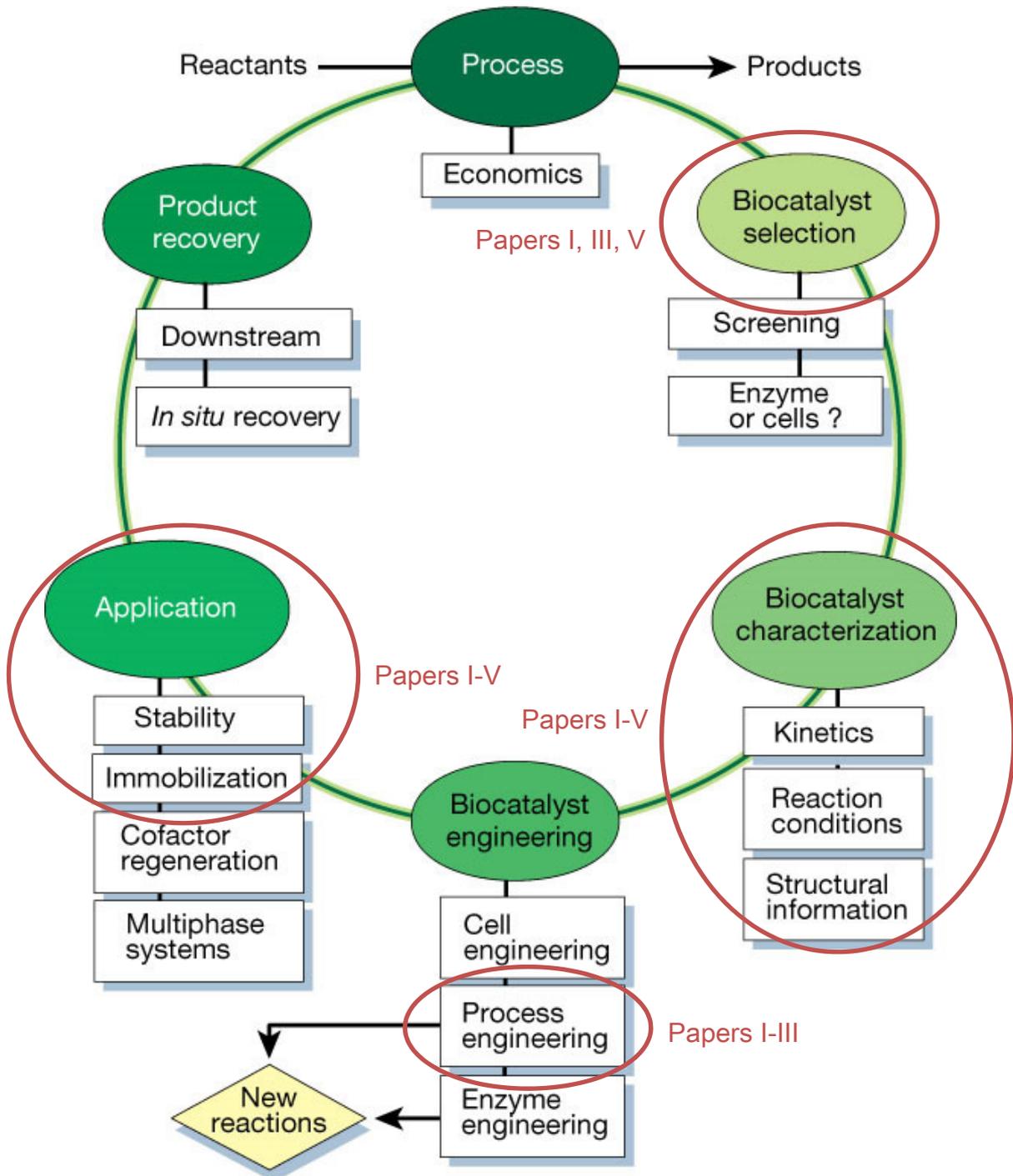


Figure 1. Illustration of the development of a new biocatalyst. Reproduced with permission from the Nature Publishing Group [8]. The red circles indicate the areas studied in this work.

The starting point of this work was the choice of the class of enzymes known as feruloyl esterases, which can modify antioxidant compounds. **The initial aim, in Paper I, was to create a biocatalyst from feruloyl esterases that provides high product yields that are maintained over several consecutive synthetic reactions.** When this study started, only a few examples of the immobilization of feruloyl esterases had been published, generally showing rapid inactivation of the enzymes, leading to poor reusability of the biocatalysts. Mesoporous materials are a new type of immobilization support with robust mechanical properties, a controllable pore size and a large pore volume allowing high enzyme loadings. The hypothesis was that immobilizing the enzyme in a mesoporous support would solve the problems associated with the inactivation of feruloyl esterases used in synthesis applications, previously reported. A protocol was therefore developed for the immobilization of feruloyl esterases in mesoporous materials (Paper I). Furthermore, immobilization itself can be seen as part of the process design, as the enzyme activity can be altered and, ideally, controlled by choosing the immobilization conditions carefully or by varying the material properties of the mesoporous silica.

Interestingly, the product selectivity of the feruloyl esterases changed upon immobilization into mesoporous silica, which gave rise to several questions and hypotheses that were addressed in the other papers: What is actually happening during the immobilization, and does this process affect the enzyme activity? Is the pH in the microenvironment inside the pores different from that in the bulk solution? Could a structural model of the studied feruloyl esterase provide additional information on how immobilization affects enzyme activity? How can it be proven that the results observed are due to the immobilization of most of the enzyme inside the pores, and not on the outer surface of the mesoporous particles? The questions that arose after interpreting the results presented in Paper I were kept in mind in the planning and execution of the studies described in Papers II, III and IV. At the same time, the different papers constituting this thesis can be seen as breaking down the challenge of a more rational biocatalyst design into problems of tangible pieces with the aim to fill the gaps in fundamental knowledge within this area.

**In the study described in Paper II, the aim was to develop a method that would allow real-time studies of the enzymes entering the pores, to provide a better understanding of the immobilization process itself and, at the same time, allowing the stability of adsorption of the immobilized enzyme to be studied.** The methods available when this

study was performed had several limitations and immobilization into the pores was often regarded as a black-box process. The method developed proved to be a useful tool for providing enzyme-specific information on both the adsorption to the support and the stability of the enzyme-support interactions.

**In an attempt to elucidate the relation between immobilization conditions and enzyme activity, the study described in Paper III was designed to answer the question of how the enzyme activity is affected by pH during the immobilization process.** Changes in enzyme activity are often explained by conformational changes of the enzyme, without any real evidence of this. The enzyme structure of the studied feruloyl esterase was therefore modeled and related to the enzyme activity. Changes in enzyme activity could also be explained by the microenvironment inside the pores differing from that in the bulk solution surrounding the mesoporous particles.

**The aim of the study presented in Paper IV was to test the hypothesis that the pH inside the pores was different from that in the bulk solution.** This required a new approach in which the enzymes were labeled with a fluorescent probe to characterize the pH in the micro-environment. Papers II-IV thus concentrate on the characterization of the biocatalyst, which is necessary to understand the observed changes in activity of the immobilized enzymes.

**The final paper, Paper V, presents a review on methods that elucidate the relation between the immobilization conditions, the microenvironment and the material properties, and enzyme activity, which can be seen as summary of the aims of this thesis.** A method of calculating the degree of pore filling was developed in order to provide a straightforward means of comparing enzyme immobilization and pore utilization in different mesoporous materials. If the methods described in Paper V are applied appropriately, it should be possible to gain a better understanding of the interactions between enzymes and support materials. Understanding the nature of these intermolecular forces has been identified as a key factor in controlling enzyme activity by immobilization [9, 10]. Two new tools were developed in this work for application in this field, namely the method of quartz crystal microbalance with dissipation monitoring (QCM-D) (Paper II) and the measurement of pH with a fluorescent probe (Paper IV). I am confident that the work presented in this thesis will be useful building block in the development of more rationally designed biocatalysts, making them a viable and competitive alternative to inorganic catalysts.



## 2 Production of antioxidant compounds using feruloyl esterases

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Certain diets rich on fruits and vegetables, are believed to be beneficial to health, partly due to naturally occurring phenolic compounds [11, 12]. These beneficial effects are often linked to the antioxidant ability of phenolic compounds, which protect the body from free radicals. However, there is also evidence of a more advanced mechanism in which phenolic compounds in the bloodstream interact in cell signaling, affecting the growth and apoptosis of cells. Hydroxycinnamic acids, which are part of these antioxidant phenolic compounds, were studied in this work, especially in the application of feruloyl esterases for their modification.

### *2.1 Antioxidant properties of hydroxycinnamic acids*

Hydroxycinnamic acids are a class of phenolic compounds that occur naturally in plant materials. They constitute the building blocks for the biosynthesis of a wide range of compounds such as lignins, flavonoids and coumarins. The hydroxycinnamic acids themselves are synthesized by the transformation of phenylalanine or tyrosine into cinnamic acid or *p*-coumaric acid, respectively, which can then be further modified into other hydroxycinnamic acids, as illustrated in Figure 2.

The interest in hydroxycinnamic acids derives from their bioactivity, which is related to their antioxidant and antimicrobial properties. Diets based on fruit and vegetables generally have high concentrations of hydroxycinnamic acids, and health benefits have been linked to the intake of these compounds [13]. Among the most studied compounds are ferulic acid, which has been proposed to have many potential therapeutic effects, such as anti-inflammatory, anticancer and neuroprotective [14, 15]. The common understanding is that these benefits arise from the antioxidant property of ferulic acid, which acts as a scavenger for free radicals. The ferulic acid radical formed is stabilized by its many resonance structures [14]. The inhibition of glycation (non-enzymatic glycosylation) and stimulation of the growth of probiotic bacteria are other suggested mechanisms behind the observed health benefits [16].

## 2.1 Antioxidant properties of hydroxycinnamic acids

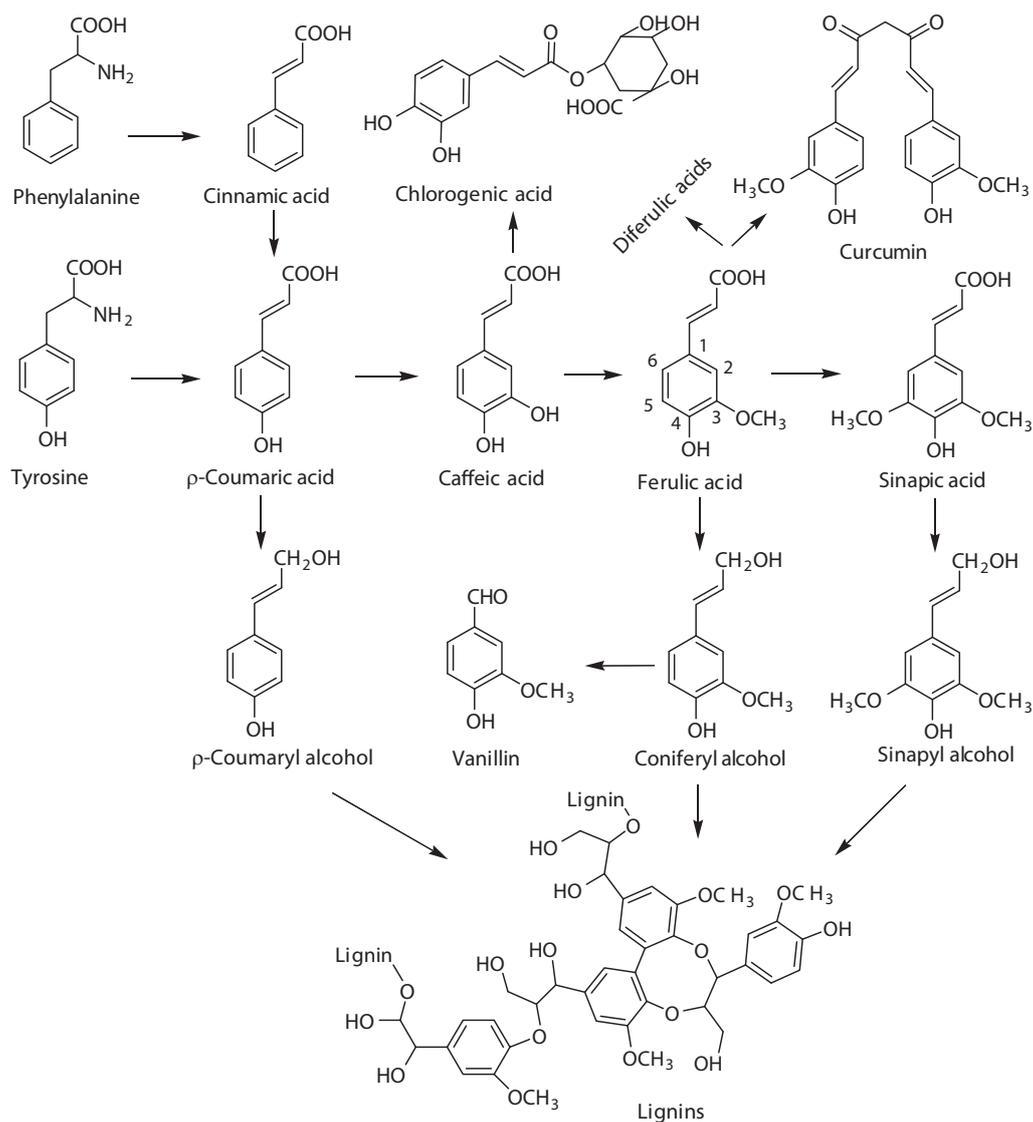


Figure 2. Molecular structures illustrating the variety of hydroxycinnamic acids and the ways in which they are related to each other. Caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid, and ester derivatives of these compounds, are the hydroxycinnamic acids most commonly modified using feruloyl esterases. The figure is adapted from [15] with permission from Elsevier.

The bioavailability, solubility and potential pharmacokinetics of the various hydroxycinnamic acids are very different [13-15]. Both the substituents on the benzene ring and any group that is bound through esterification to the carboxylic acid end group will change these properties. Generally, the antioxidant activity seems to increase with the number of substituents, but it also depends on their type and location [17]. To enhance the antioxidant ability of hydroxycinnamic acids, or to customize their solubility, some sort of modification is often necessary [18], hence the need for feruloyl esterases that are active on a broad range of hydroxycinnamic acids [19]. For a more extensive overview of bioactive ferulated compounds the reader is referred to the review by Ou and Sun [16]. When complex sugar molecules are

attached to the hydroxycinnamic acids, purification and isolation become difficult, and enzymatic synthesis offers a simpler way of obtaining the desired compounds.

## 2.2 *Modification of hydroxycinnamic acids by feruloyl esterases*

The natural degradation of ester linkages in plant cell walls is generally performed by esterases (also known as ester hydrolases), as shown in Figure 3. Feruloyl esterases (E.C. 3.1.1.73) are a subclass of carboxylic ester hydrolases, and are known to release ferulic acid and other hydroxycinnamic acids through the hydrolysis of ester linkages to hemicellulose [20]. The substrate specificity of feruloyl esterases is quite diverse. Therefore, it can be difficult to find an active feruloyl esterase for a particular hydroxycinnamic acid, as the structural variety among these compounds is broad, as shown in Figure 2. Feruloyl esterases have only been characterized relatively recently, they have thus not been as well studied as other hydrolases such as lipases [18]. The first classification of feruloyl esterases was based on the hydrolysis profile of 4 synthetic hydroxycinnamic acid methyl esters [21]. However, a new classification scheme has been proposed based on sequence-derived descriptors (patterns in amino acid composition and physico-chemical composition) and testing of the activity on an extended number of ester substrates in combination with modeling of the active site structure [19, 22]. Being able to predict the specificity allows for the selection of a suitable feruloyl esterase for a certain hydroxycinnamic acid.

The direct industrial applications of feruloyl esterases are in the degradation of biomass and the subsequent release of hydroxycinnamic acids. In the biofuel and pulp and paper industries feruloyl esterases are mainly used in hydrolysis for the debranching (cleaving side groups) of hemicellulose, thereby also breaking the linkages between lignin and hemicellulose, making the material more accessible to the other enzymes in the mixture [20, 23]. Other important areas of application are within the food processing industry and in pharmaceutical production. The bioavailability of cereals can be increased through enzymatic pre-degradation. For pharmaceuticals and cosmetic creams, the enzymes are applied in the release and subsequent purification of hydroxycinnamic acids.

## 2.2 Modification of hydroxycinnamic acids by feruloyl esterases

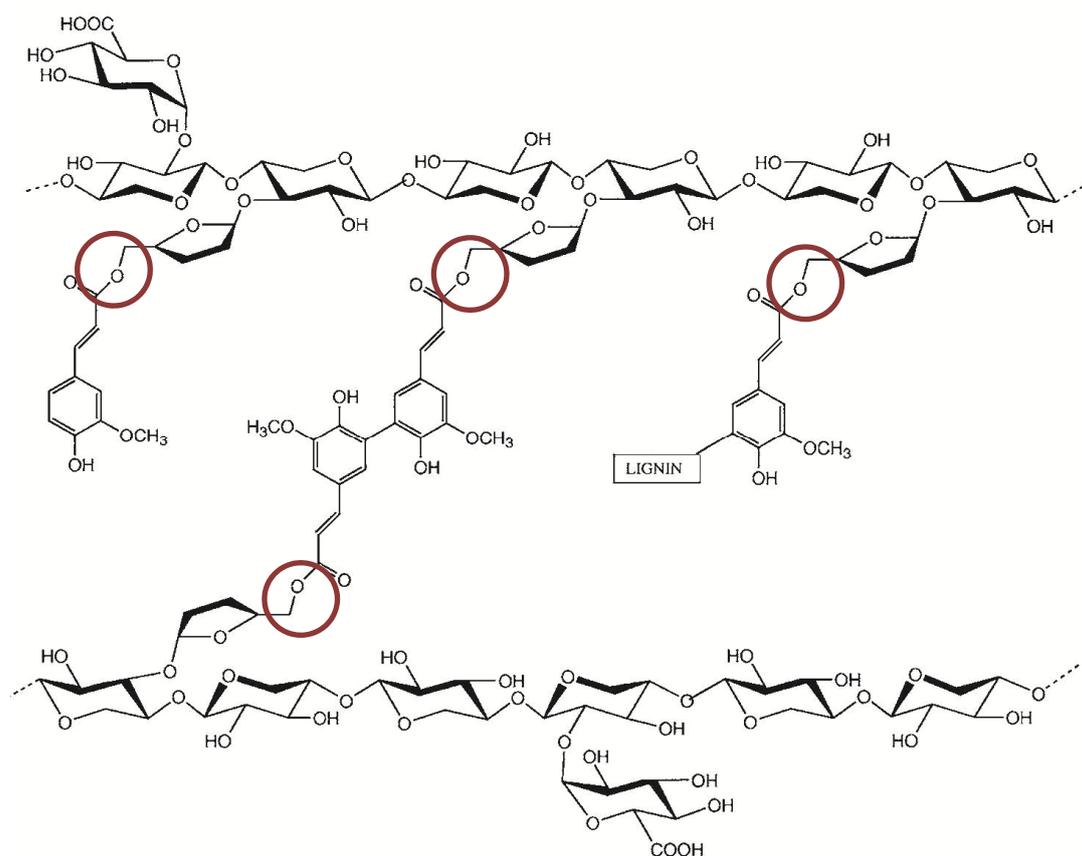


Figure 3. Hemicellulose with arabinofuranosyl residues linked to ferulic acid or diferulic acid. Cleavage sites for feruloyl esterases are indicated by the red circles. Reproduced from [24] with permission from Springer.

The natural environments of feruloyl esterases are aqueous solutions. Interestingly, under conditions of low water content, the hydrolytic activity of feruloyl esterases can be reduced or completely eliminated. The enzymes can instead be used for esterification or transesterification reactions, as shown in Figure 4, and to modify the properties of hydroxycinnamic acids. The first example of the use of feruloyl esterases for synthetic reactions was reported in 2001, when an enzyme from *Aspergillus niger* was used to esterify ferulic acid with n-pentanol in a microemulsion reaction solution [25]. Other modifications that have been demonstrated involve secondary alcohols [26, 27], glycerol [28] and di- and oligosaccharides [29-31]. Reactions involving feruloyl esterases are summarized in Table 1. In most of these examples, microemulsions with 1-3% water were used as the reaction medium, and the major fractions were two different organic solvents. There are also examples in which the organic solvents have been replaced by ionic liquids [32, 33], which allows a broader range of substrates or products to be solubilized. The general trend regarding product yields is that high yields can be achieved with modifications using alkyl alcohols, whereas the

yields obtained when using ferulated saccharides are generally lower. It can also be concluded that bulky alcohol groups on the hydroxycinnamic acid substrate reduce the transesterification yield. The main challenge when using feruloyl esterases in biocatalysis is to create conditions that favor esterification/transesterification and reduce hydrolysis, without inactivating the enzyme or reducing its stability.

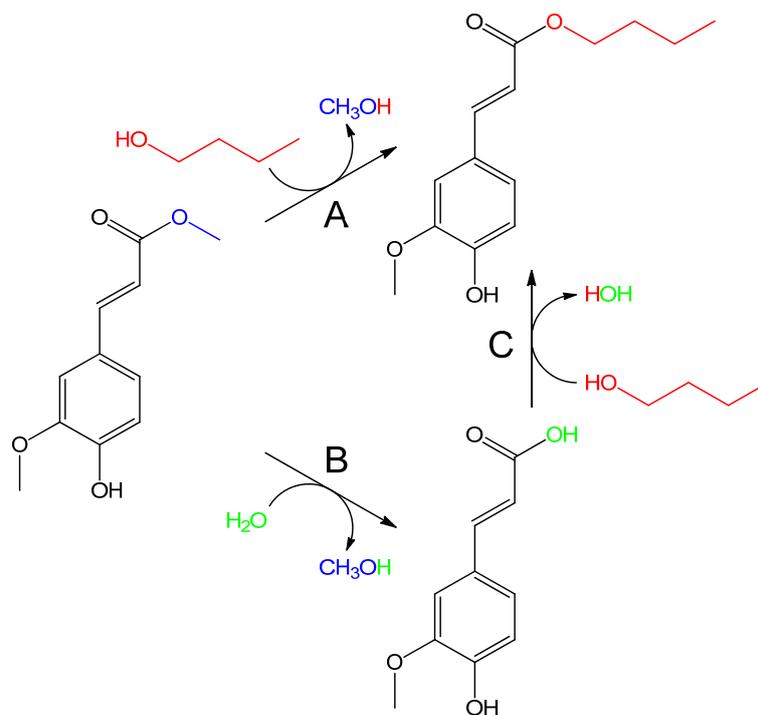


Figure 4. (A) Transesterification of methyl ferulate to butyl ferulate, (B) hydrolysis of methyl ferulate to ferulic acid, and (C) esterification of ferulic acid to butyl ferulate. The enzyme assay was performed in tubes on a shaking incubator and the product was quantified using HPLC (Paper I).

During the course of this work, the hydrophobic modification of ferulic acid to butyl ferulate [34] was studied (Figure 4A) as a model reaction to investigate the activity of feruloyl esterases in mesoporous materials (Papers I-IV). This system also allowed the substrate 1-butanol to be used as the solvent for the other substrate (methyl ferulate), which minimizes the number of components in the reaction mixture. Ferulic acid will generally be generated as a side product as it is difficult to completely suppress the hydrolytic activity of the enzyme.

Some reactions performed with feruloyl esterases (Table 1) can also be catalyzed by lipases, for example, the synthesis of ferulate sterols [35] and ferulate glycosides [36], and the esterification of various hydroxycinnamic acids with octanol [37]. The specificity of lipases is often broad, but in the case of hydroxycinnamic acids, certain configurations render low product

yields [38]. Most examples of the use of lipases in the biocatalysis of hydroxycinnamic acids involve the use of vinyl esters of hydroxycinnamic acids as substrates, which are generally not commercially available, making custom synthesis of the substrate necessary. Additionally, lipases can lose their regioselectivity at low substrate solubility for some hydroxycinnamic acids causing the formation of unwanted side products [39]. For further details on reactions catalyzed by lipases and feruloyl esterases the reader is referred to the review by Zeuner et al. [38].

Table 1. Summary of synthetic reactions catalyzed by feruloyl esterases. (Supplementary information is given in Table 2)

Product/reaction	Organism/source of enzyme	Yield <sup>a</sup> (%)	Reaction system	Ref.
Pentyl ferulate	<i>Aspergillus niger</i> (FaeA)	60	Microemulsion	[25]
Transesterification of vinyl acetate with a broad range of secondary alcohols	<i>Humicola insolens</i> (crude preparation)	52	Aqueous	[26, 27, 40]
Esterification of p-hydroxyphenyl-acetic acid with 1-propanol	<i>Fusarium oxysporum</i> F3 (Fae-II)	75	Microemulsion	[41]
Butyl ferulate Butyl coumarate Butyl sinapinate Butyl caffeate	<i>Sporotrichum thermophile</i> (StFaeC); <i>Fusarium oxysporum</i> F3 (FoFae-I)	75	Microemulsion	[30, 42]
L-arabinose ferulate	<i>Sporotrichum thermophile</i> (StFaeC)	85	Microemulsion	[30, 43]
Transesterification of vinyl ferulate with different glycosides	Different commercial enzyme preparations with both lipase and feruloyl esterase activity	60-95	A broad range of organic solvents	[36]
L-arabinobiose ferulate	<i>Sporotrichum thermophile</i> (StFaeC)	18	Microemulsion	[31]
Feruloyl D-arabinose	<i>Sporotrichum thermophile</i> (StFaeC)	45	Microemulsion	[44]
Glyceryl ferulate, glyceryl sinapinate, glyceryl coumarate	<i>Aspergillus niger</i> (Fae-PL)	80	Glycerol based	[28, 45]
Ferulated di- and oligosaccharides	Commercial enzyme preparation (Depol 740L)	27	Microemulsion	[46]
Diglycerol ferulate	<i>Aspergillus niger</i> (Fae-PL)	95	Diglycerol-based	[47]
Glycerol sinapate	<i>Aspergillus niger</i> (AnFaeA)	21	Ionic liquids	[33]

<sup>a</sup>The yields are generally calculated as the molar amount of product formed compared to the initial amount of substrate, but are sometimes reported as the fraction of the substrate converted.

As feruloyl esterases are normally active in aqueous solutions, subjecting them to other solvents is likely to affect their activity and stability [32, 48]. It is therefore important to find solvents that do not cause inactivation. Enzyme immobilization is one way of preventing enzyme inactivation, and may also lead to increased operational stability of the enzyme, as discussed in Section 3.8. At the same time, immobilization generally facilitates the reusability of the biocatalyst by facilitating separation from the reaction medium. Prior to this work, a few studies had been carried out on the immobilization of feruloyl esterases through cross-linked enzyme aggregates [32, 34, 49], which are summarized in Table 2. Although they were active, the enzymes were relatively quickly inactivated, losing most of their activity after 2-5 synthetic runs; the only stable enzyme being that immobilized in a chitin gel. The hypothesis tested in Paper I was that immobilization of feruloyl esterases in mesoporous particles would improve enzyme stability, while maintaining activity and allowing the enzyme to be reused. Continuing the work presented in this thesis, the studies described in Papers II, III and IV can thus be regarded as different approaches to increasing our fundamental knowledge on how feruloyl esterase activity is affected by immobilization in mesoporous materials.

Table 2. Immobilization of feruloyl esterases and their reusability from different solvents

<b>Immobilization technique</b>	<b>Enzyme</b>	<b>Reaction</b>	<b>Reusability</b>	<b>Ref.</b>
Cross-linked enzyme aggregates	AnFaeA from <i>Aspergillus niger</i>	Transesterification of methyl sinapate to butyl sinapate (other butyl esters synthesized at lower yields)	No retained activity after two 96-h runs in n-hexane/1-butanol/water (47.2:50.8:2.0 v/v/v)	[34]
Cross-linked enzyme aggregates	Ultraflo L Depol 740L Depol 670L	Transesterification of methyl ferulate to butyl ferulate	No retained activity after two 6-day runs in n-hexane/1-butanol/water (47.2:50.8:2.0, v/v/v)	[49]
Cross-linked enzyme aggregates	AnFaeA from <i>Aspergillus niger</i>	Esterification of sinapic acid with glycerol to glycerol sinapate	No retained activity after five 2-h runs in the ionic liquid [C <sub>5</sub> O <sub>2</sub> mim][PF <sub>6</sub> ] with 2.5 M glycerol and 15% water	[32]
Cross-linked enzyme aggregates	Fae-II from <i>Aspergillus awamori</i>	Release of ferulic acid from ethyl ferulate	Not measured	[50]
Chitin-gel (Chitopearl BCW3003)	Pectinase PL “Amano” preparation containing feruloyl esterases	Esterification of ferulic acid to glycerol ferulate	Stable after five 23-h runs in glycerol with 2% water	[51]



### 3 Enzyme immobilization in mesoporous materials

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The obvious question when first studying this topic is why it is necessary to increase the cost and complexity by immobilizing enzymes. In this chapter I will outline why mesoporous materials are useful for enzyme immobilization, and explain how the immobilization process can be characterized in some detail, as there are special considerations when using mesoporous particles, as discussed in Section 3.2. The results and methods discussed in this chapter should, however, be seen in the light of Chapter 4, where changes in the activity of the enzymes upon immobilization are discussed. Both the immobilization material itself and the conditions during immobilization can alter the specific enzyme activity. Hence, knowledge of the immobilization process provides a basis for the better understanding of the changes in enzyme activity upon immobilization. In order to create a robust biocatalyst, not only should the activity of the immobilized enzymes be high for the desired reaction over several runs, but it is also important to achieve a high pore loading so as to utilize the whole pore volume efficiently, and thus minimize the amount of support material.

#### *3.1 Why immobilize enzymes and what difficulties are encountered*

It is generally difficult to separate free enzymes from a reaction solution, which limits their reusability making complex product purification schemes necessary. Enzyme retention and long-term operational stability must also be considered when using continuous reactions and in industrial use, as enzymes are generally expensive to produce, substantially increasing the cost of the processes in which they are used. When performing enzymatic reactions in organic solvents, which is often necessary for hydrolases to exhibit their biosynthetic behavior, the reusability of the solvent must also be considered to avoid expensive and unsustainable waste handling. The immobilization of enzymes simplifies the separation of the enzymes from the solvents, while at the same time the enzyme stability is generally increased. Furthermore, easy enzyme recovery also simplifies product purification. The reason for enzyme immobilization is to create a biocatalyst that retains a high catalytic activity, giving high product yields over several reaction cycles.

Many enzymes are classified as allergenic as they can penetrate the skin [52]. This is important as many people are exposed to enzymes, for example, when handling detergents.

### 3.1 Why immobilize enzymes and what difficulties are encountered

Immobilizing the enzyme on a solid support reduces the risk of skin penetration as the immobilization support particles are generally several times larger than the enzyme molecules. However, some inactivation of the enzymes during immobilization can be expected, as the binding or adsorption of the enzyme to the support may result in structural changes rendering the enzyme inactive, or re-orientation of the enzymes such that the entrance to the active site is blocked. Table 3 lists the main advantages and disadvantages of enzyme immobilization.

Table 3. Advantages and disadvantages of different immobilization strategies [53-56]

<b>Immobilization in general</b>	<b>Advantages</b>	<b>Disadvantages</b>
	The enzymes can be recovered	Potential diffusion limitations
	Downstream processing is easier as the product stream does not contain enzymes	Costs involved in the synthesis of the supporting material and in the enzyme immobilization process
	Can improve overall enzyme stability by stabilization of enzyme structure	Enzymes may be inactivated during the immobilization process
	Possibility of continuous reactions as recovery of enzymes is easier	
<b>Type of immobilization</b>	<b>Advantages</b>	<b>Disadvantages</b>
Binding to support	Easy separation of support particles from reaction solution (if particle size is not too small)	Reuse of the support material is difficult if the enzyme is inactivated
	The choice of support material and its surface properties can be used to control enzyme activity and stability	Most of the biocatalyst weight/volume consists of the support material and not the active enzyme
	Well established with several industrial applications	If the enzyme is not covalently bound to the support, enzyme leakage may be a problem
Entrapment in gels, capsules or membranes	Protective environment for the enzyme compared to the outer surface of an immobilization support	Can be difficult to find gelling or encapsulation conditions under which the enzymes remain stable
	The hydration of the enzyme can be controlled to a larger extent	Diffusion of substrate and product may be hindered
Cross-linked enzyme aggregates	Lower cost as there is no need for the synthesis of a support material	Optimal aggregation conditions are highly dependent on the enzyme
	Insoluble aggregates allow for easy enzyme recovery and increased stability	Glutaraldehyde can cause inactivation of some enzymes if certain amino groups are cross-linked

Although immobilization adds another level of complexity to the process, it also alleviates several of the problems encountered when running processes with free enzymes, and should, therefore, reduce the overall cost. Extensive research has been carried out on enzyme immobilization over recent decades, however, a number of problems still remain to be solved. There is considerable variation in the optimal immobilization conditions, with regard to catalytic activity, between different enzymes. Therefore, a trial-and-error approach to process design is often necessary for the development of a new biocatalyst, despite the fact that this is time consuming and expensive. Establishing the right immobilization conditions while maintaining catalytic activity is not always straightforward, as many factors influence both immobilization and activity. It is also often necessary to tailor the support material to the enzyme, although the synthesis of the material should preferably be as simple as possible.

The nomenclature used for various enzyme immobilization strategies in the literature is inconsistent, resulting in number of different categories [53, 54, 57]. However, three main categories can be distinguished: enzyme binding to the surface of a solid support; entrapment of the enzyme in a polymer gel or matrix, and cross-linking of the enzymes themselves to form aggregates, as illustrated in Figure 5. The number of categories can be expanded depending on the type of interactions responsible for binding the enzymes to the support or the type of entrapment. It is also possible to combine different approaches such as the cross-linking of enzymes on a porous solid support to prevent leakage.

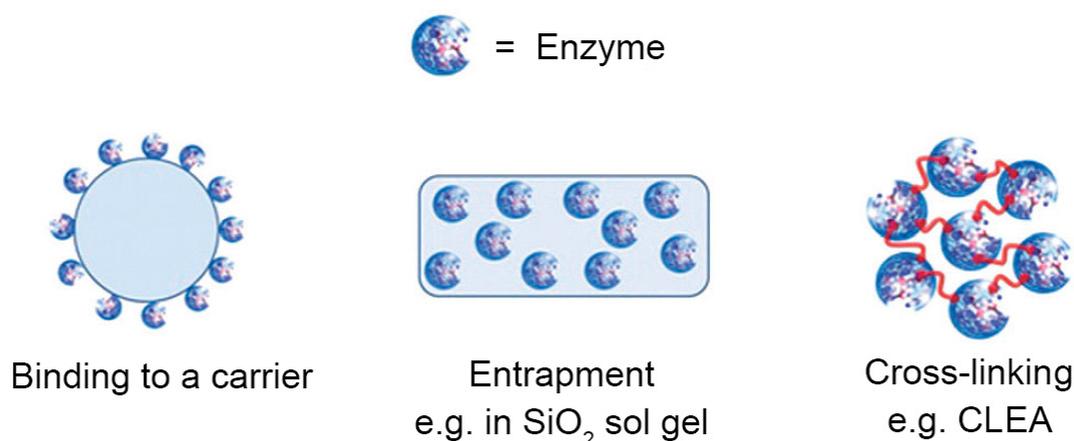


Figure 5. Three different approaches to enzyme immobilization. Figure reproduced from [52] with permission from the Royal Society of Chemistry.

### 3.2 Why use mesoporous materials as an immobilization support?

#### 3.2 *Why use mesoporous materials as an immobilization support?*

A relatively new type of immobilization support, mesoporous materials, was studied in this work. The main feature of these materials is their porous structure (pore diameter 2-50 nm), which allows for high enzyme loadings, while at the same time providing a protective environment for the enzymes. Table 4 lists the advantages and disadvantages of mesoporous materials. Immobilization of enzymes in a mesoporous support (or carrier) is sometimes referred to as encapsulation or entrapment, while others simply refer to it as normal binding to a support (through any type of molecular interaction) [54, 55]. In this thesis, the whole process of the enzyme molecules entering the pores and their subsequent adsorption onto the pore walls will simply be referred to as immobilization.

Table 4. Advantages, disadvantages and other considerations in enzyme immobilization in mesoporous materials, compared to other kinds of support

<b>Material-related properties</b>	<b>Advantages, disadvantages and other considerations</b>
Porous structure	<p>Large surface area allows for the efficient utilization of the support material in relation to its weight.</p> <p>Offers a protective environment for the enzymes inside the pores, similar to the enzyme being encapsulated in a gel.</p> <p>The synthesis of a porous structure is generally more expensive than, for example, solid beads.</p> <p>Some mesoporous materials are not commercially available, and in-house synthesis and access to equipment for material characterization are thus required.</p> <p>Large particle sizes may lead to some of the pore volume not being filled.</p> <p>Substrate/product diffusion inside the pores may be limited.</p>
Controllable pore size and well-ordered structure	<p>The pore size can be adjusted to match the size of the enzyme.</p> <p>Molecular crowding inside the pores and more molecular interactions with the support due to the curvature of the pore wall may increase or decrease enzyme activity (see Paper V).</p>
Particle size/particle morphology	<p>The length of the pores and the pore size may influence the specific activity of the immobilized enzyme.</p>
Stable material	<p>The material will always be mechanically stable at the conditions (pH, temperature, mechanical stress) under which the enzyme is catalytically active.</p>
Surface modifications	<p>Silica surfaces can easily be functionalized or modified to adjust properties such as surface charge or hydrophobicity.</p>
Material synthesis prior to enzyme immobilization	<p>Synthesis conditions are not dictated by the conditions that are suitable for the enzymes, which allows for a broader range of possible materials.</p>

Different types of mesoporous materials have been studied, of which Santa Barbara Amorphous material number 15 (SBA-15) is the most commonly used for enzyme immobilization. SBA-15 is characterized by high mechanical and thermal stability, while the pore size range and particle size can be varied in a relatively broad range [58]. Mesoporous materials have been successfully used as immobilization supports for a broad range of enzymes [9, 59]. Many areas of application of biocatalysts have been identified, for example, as biosensors, in food processing, the production of pharmaceuticals, in biorefineries and biofuel production [60]. However, prior to the present work there were no reports of the immobilization of feruloyl esterases in mesoporous materials. The mesoporous materials studied in this work are listed in Table 5.

Table 5. Characteristics of the different mesoporous materials used in this work

<b>Material</b>	<b>Pore diameter (nm)</b>	<b>BET<sup>c</sup> surface area (m<sup>2</sup>/g)</b>	<b>Pore volume (cm<sup>3</sup>/g)</b>	<b>Paper</b>
SBA-15 <sup>a</sup>	5.0	924	0.73	I
SBA-15 <sup>a</sup>	9.3	528	1.17	I
HMM <sup>b</sup>	9.4	463	0.91	II
SBA-15 <sup>a</sup>	7.8	679	1.17	III and IV

<sup>a</sup>Santa Barbara Amorphous material number 15, with a well ordered hexagonal pore structure, and a defined pore size distribution.

<sup>b</sup>Hiroshima mesoporous material with non-ordered structure and wider pore size distribution than SBA-15.

<sup>c</sup>BET: Barret-Joyner-Halenda theory

The synthesis of most ordered mesoporous materials, including SBA-15, is illustrated in Figure 6. A block copolymer surfactant acts as a template by forming micelles. This is followed by the addition of tetraethyl orthosilicate (TEOS) as a silica precursor which, upon hydrolyzation, initiates the condensation of a silica oxide network around the template, and induces the transition of micelles into rods that flocculate forming a hexagonal structure [61]. Finally, the template is removed through calcination (by heating in an oven at high temperatures). Paper II describes another type of mesoporous particles, namely Hiroshima mesoporous materials (HMM). The main characteristics of HMM are their very small particle size, around 50 nm (compared to 1-2  $\mu\text{m}$  for SBA-15), and their non-organized porous structure. The latter is the result of the method of synthesis, which differs in that an oil-in-water emulsion consisting of water, octane, styrene and TEOS is used, and the particles are formed by the polymerization of styrene and TEOS.

### 3.3 Choice of immobilization strategy

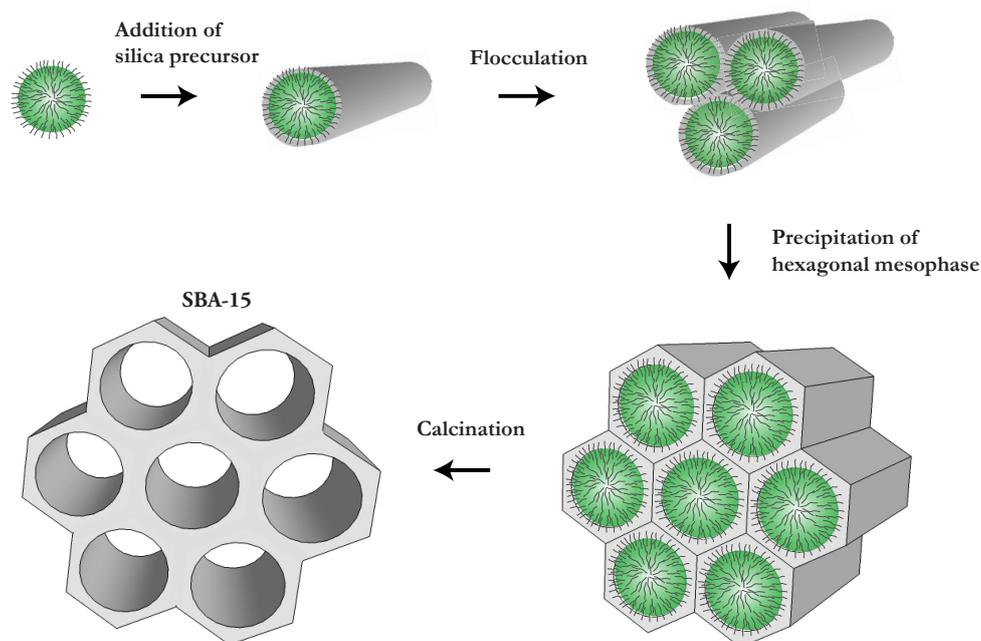


Figure 6. The current understanding of the synthesis of SBA-15, where the addition of silica to the template micelles induces the formation of cylindrical silica pores that flocculate forming hexagonal structures, and the removal of the template through calcination. Figure reproduced from Hanna Gustafsson [62] with permission.

The structure of the material is normally characterized by small-angle X-ray scattering and transmission electron microscopy. The pore volume, pore diameter and surface area are generally determined by the adsorption (physisorption) of nitrogen gas and application of the gas law in combination with Barret-Joyner-Halenda theory. For a more detailed description of the materials and the characterization methods used in this work, the reader is referred to Paper V.

### 3.3 Choice of immobilization strategy

Having chosen the type and material for immobilization, the question of how the enzyme should be attached to the support must next be addressed. The enzyme can either be physically adsorbed or covalently bound to the support surface, as illustrated in Figure 7. Physical adsorption relies on molecular interactions, which may be any combination of van der Waals, hydrophobic or ionic interactions. In practice, immobilization is generally performed by simply mixing the support particles with the enzyme in a solution. The disadvantage of this simple process is that the interactions may be weak, resulting in desorption or leakage of the enzyme from the support material [55, 59]. This problem can be solved by covalently binding the enzyme to the support. Any interactions in which an enzyme is

involved can cause changes in its tertiary structure, and may therefore affect both the stability and activity of the enzyme. In this work, physical adsorption was chosen as the immobilization strategy as this approach is the most straightforward, as no modification of the enzyme or the support was required. The adsorption process itself is often treated as a black box, where active enzymes and high product yields after immobilization are considered to provide sufficient information. Therefore, to expand current knowledge on how enzymes are immobilized in mesoporous materials, a method was developed for real-time studies of this process, which also allows leakage of the enzyme from the support material to be studied in more detail. This is described below in Section 3.4.

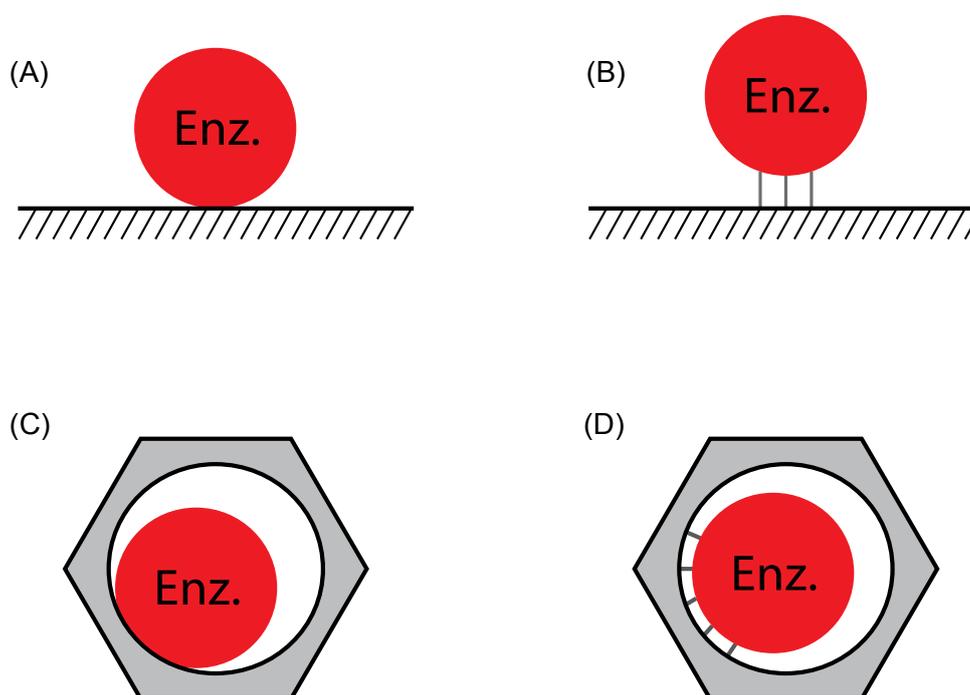


Figure 7. Schematic representation of (A) physical adsorption and (B) covalent binding of the enzyme to the surface of a flat support. In theory, more interaction points are generated when the enzyme is immobilized inside the pores through (C) physical adsorption and (D) covalent binding. Typical enzyme dimensions are on the order of 5-8 nm, while the pore size is usually chosen to be a few nm larger than the enzyme.

In the case of mesoporous materials, the size of the pores is often of the same magnitude as the enzyme, which means that the curvature of the pore will allow for more interaction points (Figure 7C,D). This means an increase in the probability of the enzyme somehow being affected by immobilization. It has been reported that the specific enzyme activity (i.e. the activity per unit weight or volume of the enzyme) may increase or decrease upon immobilization [59]. Similar trends were seen in the present work (Paper I), where the pore size was found to influence the specific activity of the enzyme. This was explained by differences in curvature at the enzyme adsorption site. Ideally, with full knowledge of how these interactions

### 3.4 Studying enzyme immobilization – in real time

affect enzyme activity, the support material could be used as a means of controlling the enzyme activity [55, 63]. Multiple interaction points may also stabilize the structure and allow working temperatures at which the immobilized enzymes exhibit higher activity, but where the free enzymes would quickly be inactivated.

#### *3.4 Studying enzyme immobilization – in real time*

The immobilization process is usually studied by collecting samples of supernatant and measuring the protein concentration in order to calculate the loading of enzymes in the support material [52]. For enzyme mixtures, it may be more appropriate to measure the retained enzyme activity in the supernatant, as immobilization may be selective towards certain proteins. Both these methods measure the amount of immobilized enzyme indirectly, assuming that all the enzymes removed from the solution must have been immobilized on the support. Measuring the activity retained in the supernatant also assumes that the support did not affect the activity of the non-immobilized enzymes still in solution. In the case of feruloyl esterases, most of the enzyme molecules are immobilized within the first few minutes, as can be seen in Figure 8. As the procedure normally involves centrifugation, it is not possible to follow the concentration in real time. In order to gain a deeper understanding of the immobilization process it is desirable to be able to study it on a time scale of minutes or seconds.

Quartz crystal microbalance with dissipation (QCM-D) is a surface-sensitive technique that can be used to analyze mass adsorption onto a surface (a more detailed introduction to this technique is given in Paper II). An alternating electric current is applied to a quartz crystal causing it to vibrate, and the frequency of vibration decreases as material accumulates on its surface. Dissipation monitoring is used to analyze the viscoelastic properties of the adsorbed layer: an increase in dissipation indicating a more flexible layer, less tightly bound to the surface. QCM-D is commonly used to study the formation of films, and it was hypothesized that it could also be applied to study adsorption in porous particles, allowing real-time studies of enzyme immobilization. To the best of the author's knowledge, only porous films [64-66] and not porous particles have been studied by QCM-D, and no real-time monitoring of the immobilization process in mesoporous materials had been reported prior to the work presented in Paper II.

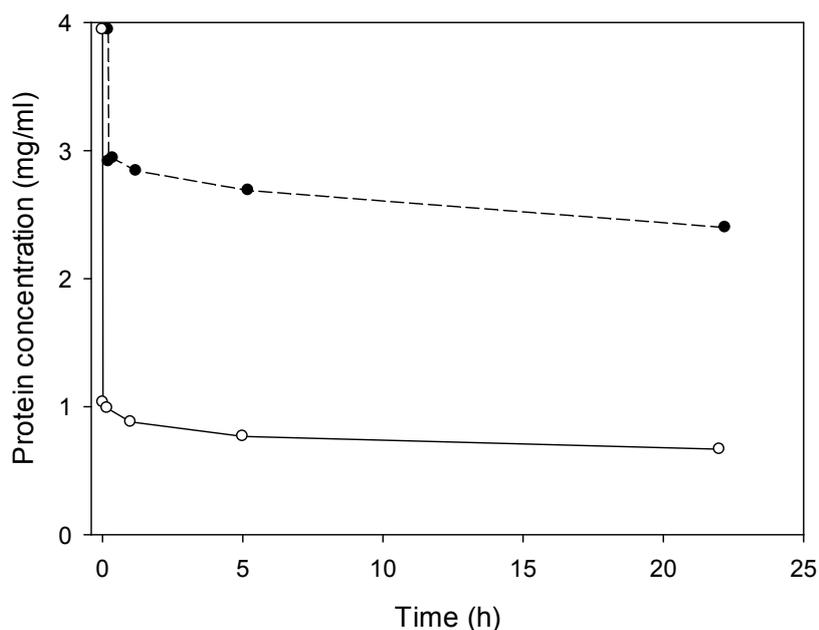


Figure 8. Protein concentration in the supernatant during the immobilization of a feruloyl esterase preparation (Depol 740L) in SBA-15 with two different pore sizes: 5 nm (●) and 9 nm (○). The graphs have been time shifted slightly to avoid overlap. Adapted from Paper I.

A protocol was developed for the binding of mesoporous particles (HMM) to the crystal, which allowed enzyme immobilization to be studied in real time. Figure 9 shows the change in vibrational frequency of the crystal and the change in dissipation as a function of time, i.e., as more particles are adsorbed onto the surface of the crystal. This technique proved to be useful in comparing the immobilization conditions of different enzymes, as described in Section 3.7. QCM-D measurements could also be used to study the stability of the immobilized enzymes, as the desorption of small amounts of enzymes were easily detected (see Section 3.8). This is not possible with traditional protein concentration measurements, as the low concentrations are close to the detection limit where noise becomes dominant.

The dissipation data recorded during the QCM-D measurements also provided novel information on the immobilization process, showing that the process consisted of two stages; initial adsorption on the outside of the particles followed by filling of the pores. This is discussed in more detail in Section 3.6.

### 3.5 Calculation of pore filling for comparison of immobilization in different materials

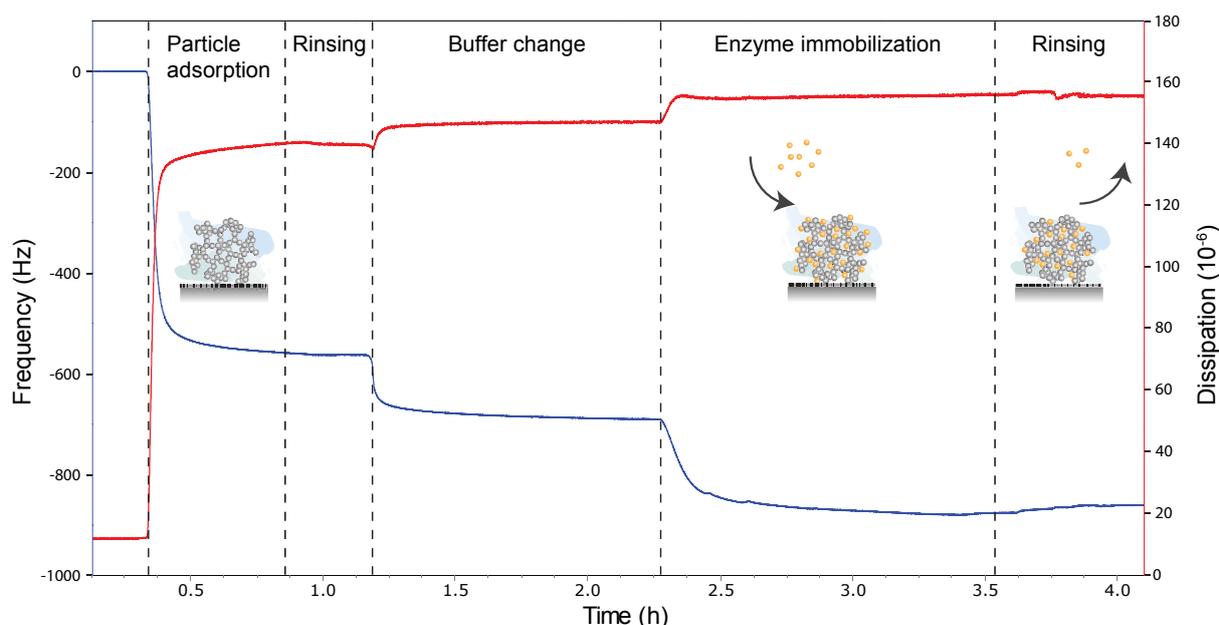


Figure 9. Results from QCM-D measurements showing the decrease in frequency (blue) and the increase in dissipation (red) over time. This illustrates the binding of the HMM particles to the crystal, followed by rinsing and changing of the buffer. Thereafter, the immobilization of the enzyme (yellow particles) can be followed in real time. Finally, the stability of immobilization was investigated by rinsing with buffer. The figure is based on data given in Paper II.

It should be possible to apply the real-time approach developed in this work to other enzymes and immobilization supports, as long as it is possible to bind the support material to the QCM-D crystal. A method of estimating the pore volume of porous particles on the QCM-D crystal was also developed as it is important to know the pore volume when comparing enzyme immobilization on different types of particles. This further expands the applicability of the method.

### 3.5 Calculation of pore filling for comparison of immobilization in different materials

The degree of enzyme immobilization, or enzyme loading, is often quantified by calculating the weight (or amount) of enzyme per unit weight of the support material. Another option is to determine the immobilization efficiency by comparing the specific activity of the immobilized enzymes to that of free enzymes. However, neither enzyme loading nor immobilization efficiency provides any information on the extent to which the theoretical pore volume of the support is filled with enzymes. Without this information it is difficult to compare immobilization in different mesoporous materials. When comparing the immobilization of different enzymes it is also appropriate to consider the volume occupied by the enzyme and

not only its molecular weight. This called for a method of calculating what was defined in this work as pore filling.

The pore filling, i.e., how much of the pore volume is occupied by enzymes,  $P_f$ , was calculated based on some simple assumptions. Firstly, it was assumed that the amount of enzyme on the outer surface of the particles could be neglected, as the outer surface only accounts for only 1-2% of the total surface area of the particles [67]. Secondly, the whole pore volume is assumed to be accessible to the enzymes. The following are required in the calculation: the dimensions of the enzyme (or its hydrodynamic radius,  $r_{prot}$ , assuming that the enzyme molecule can be approximated by a sphere), the molecular weight of the enzyme,  $M_{prot}$ , and the normal protein loading,  $P_{LD}$ , defined as  $m_{prot}/m_{mesoporous\ silica}$ , and based on indirect protein concentration measurements during immobilization (as described in Section 3.4). The density of the silica pore walls,  $\rho_{silica}$ , and the specific pore volume,  $v_{pore}$ , which is normally obtained from the characterization of the material after material synthesis, are also required. The density of the particles,  $\rho_{part}$ , can be calculated from the density of silica and the specific pore volume. This gives the following expression for the pore filling, as derived in Paper V:

$$P_f = \frac{4\pi \cdot r_{prot}^3 \cdot N_A \cdot P_{LD} \cdot \rho_{part} (v_{pore} + \rho_{silica}^{-1})}{3 \cdot M_{prot} \cdot v_{pore}}$$

An important consideration is that 100% pore filling of can never be achieved, as the theoretical maximum close packing of enzymes assumed to be spheres in a cylinder corresponds to 67% of the total volume [68]. More irregular shapes of the enzyme molecules will lower this value.

The pore filling for the feruloyl esterase C from *Fusarium oxysporum* (FoFaeC) (simplified as a cube with the dimensions 5 nm x 6 nm x 8 nm) was calculated and found to be 2.5-6.5%, depending on the immobilization conditions (the variation in the amount of FoFaeC immobilized as a function of pH is described in Section 3.7). At 6.5% pore filling, 81% of the amount of enzyme added was immobilized, and no attempt was therefore made to optimize the amount of immobilized FoFaeC in this work, apart from varying the pH. However, it is reasonable to assume that the pore filling of FoFaeC could have been increased, by simply increasing the enzyme concentration during immobilization. Paper V presents values of pore

filling calculated using data from several already published studies. Many of the values were below 5% (Paper V), and it could be questioned whether most of the enzymes are actually inside the pores or simply on the surface of the particles. Furthermore, the concept of a “confining effect” of the pores on the immobilized enzymes can also be questioned if there is no evidence that the enzyme is actually adsorbed inside the pores. Two possible explanations of why the enzyme is only adsorbed on the outer surface are blocking of the pore entrance, or too large a molecular size of the enzyme compared to the size of the pores.

It has also been shown that the specific activity of immobilized enzymes is affected by pore loading, and this was partly explained by differences in molecular crowding in the pores [69-71]. Molecular or macromolecular crowding is usually attributed to functional changes in proteins and enzymes caused by high local concentrations, which is the case in most biological cells. It has been suggested that molecular crowding was the cause of a factor of 2 higher specific activity of organophosphorus hydrolases at high protein loadings, than at low protein loadings [70]. In contrast, the protein loading of lipases in three different materials (HMM, particle size 40 nm, SBA-15 particle size 300 nm, and SBA-15 particle size 1000 nm) showed almost identical results, while the specific activity differed by factors of 2-3, and the pore filling varied from 2.3 to 3.7% ([72] and Paper V). These results indicate that there is no correlation between pore filling and specific activity, and that the particle size governs the specific activity.

The mechanisms by which molecular crowding in the pores alters the specific activity are still the subject of much speculation, but when discussing molecular crowding, it is reasonable to compare the results in terms of pore filling in order to avoid misinterpreting the results when the enzymes are actually located on the outer surface of the particles. Higher transesterification activity was seen when the feruloyl esterases were immobilized in particles with a larger pore size (9 nm) than in those with a smaller pore size (5 nm), where most of the enzymes were assumed to be on the outer surface of the particles as the pores were too small for the enzymes to enter (Paper I). It appears that feruloyl esterases prefer to be in a less crowded environment, as the specific activity of the enzymes in the particles with the larger pore size was higher. In this case, there is more available volume around each enzyme molecule as the whole pore volume is accessible.

### 3.6 Proof that the enzymes are located inside the pores

The fact that there is often insufficient proof that the enzymes are actually inside the pores has been identified as a problem that must be solved, suggesting new tools are needed for the characterization of systems with enzymes in mesoporous particles [73]. The problem is that in most cases, the interpretation of the results, speculations and the conclusions are based on the assumption that most of the enzyme molecules are located inside the pores, although there is no proof that this really is the case. It can be questioned whether the enzymes really are inside the pores at low values of pore filling. The enzymes may, in fact, be partially or completely adsorbed on the surface, as illustrated in Figure 10. If the total pore volume were filled with enzymes, the amount adsorbed on the outer surface of the mesoporous particles could be neglected.

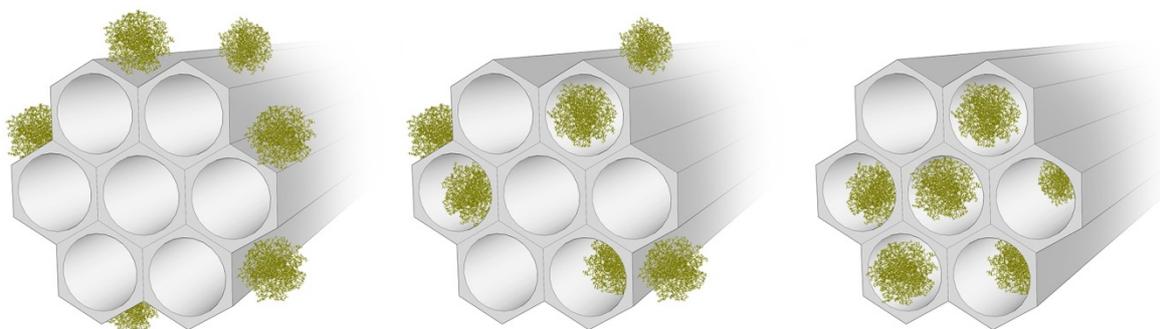


Figure 10. Illustration of three possible distributions of enzymes on mesoporous particles: immobilization only on the outer surface (left); a combination of immobilization on the outer surface and inside the pores (center), immobilization only inside the pores (right).

The most obvious, though indirect, evidence that the enzymes are located inside the pores has generally been that the amount of enzyme immobilized on mesoporous particles was significantly higher than that immobilized on solid particles of the same size. More than twice the amount of enzyme was immobilized in the particles with the larger pore size (73 mg enzyme/g mesoporous silica) than in non-porous particles (31 mg enzyme/g non-porous silica) (Paper I). Furthermore, the particles with the smaller pore size (assumed to be too small for the enzymes to enter) showed very similar enzyme loading to the non-porous particles. This is a clear indication that the enzymes do not enter the smaller pores during immobilization, while they do enter the larger pores (Figure 8), although it does not provide unquestionable proof.

Immobilization only on the outer surface (left); a combination of immobilization on the outer surface and inside the pores (center), and immobilization only inside the pores (right).The

### 3.6 Proof that the enzymes are located inside the pores

same trend, of higher loading in the porous particles, was observed in the QCM-D measurements (Paper II), where the enzyme loading was more than 4 times higher than the loading on non-porous particles (Figure 11). The dissipation data obtained from the QCM-D measurements provide additional information that indicates immobilization inside the pores. The increase in dissipation (Figure 11) can be divided into two stages, suggesting that the immobilization is sequential. The first stage is interpreted as immobilization on the outer surface, with an increase in dissipation due to the formation of a bulky enzyme layer. In the second stage, the dissipation is more stable, which suggests that the enzyme molecules are entering the pores. This conclusion is based on the assumption that enzymes inside the pores do not contribute to the overall dissipation, but should still contribute to the decrease in frequency. This is in line with other theories suggesting coverage of the outer surface followed by pores being filled with enzyme [74]. An additional indication that the enzymes are located inside the pores is the higher retention (percentage of enzymes still adsorbed to the particles) of enzymes immobilized in porous particles than on the flat surface (see Figure 15 and Section 3.8). If the enzymes were on the outside of the particles, they would easily desorb as from the flat surface.

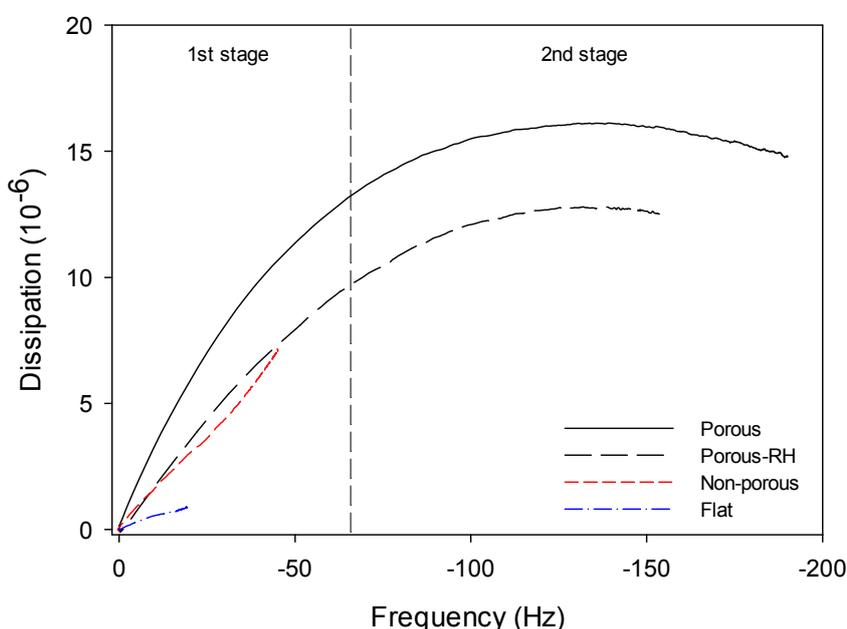


Figure 11. Results of QCM-D measurements on the immobilization of FoFaeC on different silica particles and a flat surface (Porous = HMM particles; Porous-RH = Rehydroxylated HMM particles; Non-porous = non-porous silica particles; Flat = flat silica surface). The frequency decreases as the mass of the enzyme increases, i.e. as more enzyme is immobilized on the various materials. The 1st stage is interpreted as immobilization on the outer surface of the particles, and the 2nd stage as immobilization in the pores when the increase in dissipation has flattened out. (The figure is based data given in Paper II.)

It is possible to prove mathematically that the enzyme molecules enter the pores by plotting the number of enzyme molecules per particle as a function of particle diameter on logarithmic scales, as shown in Figure 12. Data from the literature were compiled to create such a plot (Paper V). A slope of 2 indicates that the enzyme molecules are located only on the outside of the particles, whereas a slope of 3 indicates that the enzyme molecules are located only inside the pores. The slope of the log-plot in Figure 12 is 2.5, indicating that the enzyme molecules are distributed both on the surface and in the pores of the particles. The slope appears to be steeper for smaller particle sizes, which suggests that the pore volume is used more efficiently the shorter the pores are, indicating that it is difficult for the enzymes to reach the middle of longer pores.

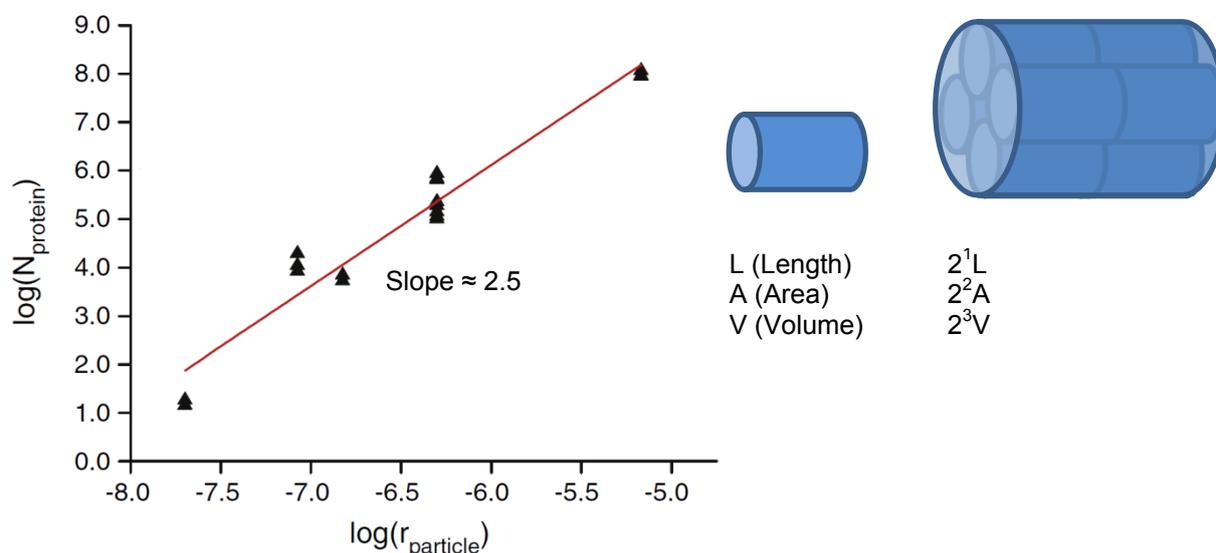


Figure 12. Left: The number of protein molecules ( $N_{\text{protein}}$ ) per support particle as a function of particle radius ( $r_{\text{particle}}$ ). The slope defines the geometrical dependence of the immobilization, i.e. whether the enzyme is adsorbed on the surface or in the pores. The data are based on the calculations presented in Paper V. Right: Visualization of how area and volume scale if the length is doubled.

In Paper V, other means of proving that the enzyme molecules are located inside the pores are summarized. One sophisticated approach involves labeling the immobilized enzyme molecules with gold-conjugated antibodies, which are then detected by slicing the mesoporous materials into thin layers and imaging them with transmission electron microscopy [75]. Based on the above, there seems to be extensive evidence that the enzymes are adsorbed inside the pores. However, at low enzyme loadings it is reasonable to assume that most of the enzyme is located on the outer surface of the particles.

### 3.7 Enzyme-dependent effects of pH on immobilization

Several factors related to enzyme immobilization are affected by changing the pH of the enzyme solution. The protonation of the hydroxyl groups at the silica surface and the surface charge of the enzyme itself are affected most directly. This means that the electrostatic interaction will vary depending on the pH, and thus the amount of enzyme immobilized. However, in the case of the feruloyl esterase FoFaeC, the relationship is the opposite to that which could be expected (Paper III). If electrostatic interactions were dominant during the immobilization process, the highest loading would be seen at the lowest pH, where the net charge difference between the surface (negative) and the enzyme (positive) is greatest. The results presented in Paper III instead show an increase in enzyme loading with increasing pH, as shown in Figure 13. High amounts of immobilized enzymes are often found at a pH around the isoelectric point (pI) of the enzyme [59, 73, 76]. This is explained by the minimization of enzyme-enzyme repulsion, which increases the relative influence of the surface-enzyme interactions, which could also provide a reasonable explanation of the higher amount of FoFaeC immobilized at higher pH (pI 6.8 [77]). It could also be speculated that hydrophilic and hydrophobic interactions play a greater role for FoFaeC closer to the pI.

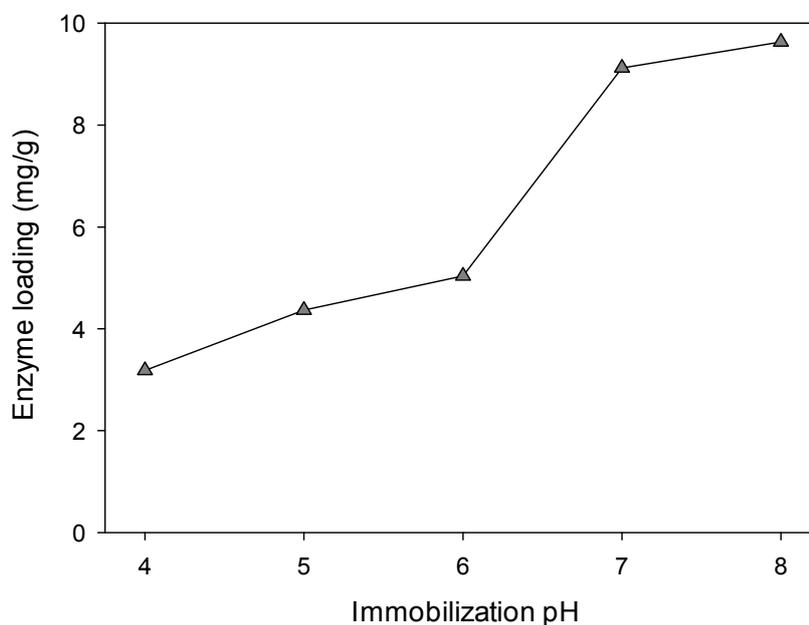


Figure 13. Enzyme loading as function of pH for the immobilization of FoFaeC in SBA-15 (9 nm pore size) expressed as weight of enzyme per weight unit of mesoporous silica. Citrate phosphate buffer was used to control the pH. (From Paper III.)

The QCM-D measurements at different pH showed the same dependence of immobilization on pH (see Figure 14). However, the same measurements with a lipase from *Rhizopus oryzae* (ROL), showed the opposite pH dependence, despite the fact that its pI (7.6 [78]) is similar to that of FoFaeC. In this case, electrostatic enzyme-surface interactions seem to be more important than minimizing enzyme-enzyme repulsion. Immobilization is evidently highly dependent on the enzyme, and further studies are required on the distribution of charge and hydrophobicity on the surface of the enzymes. Two enzymes with a high pI, a cytochrome c with a pI of 10.7 and a xylanase with a pI of 9.0, have totally different charge density and distribution of charged amino acids on the surface, and subsequently exhibit different immobilization behavior [79]. Therefore, the surface charge distribution of FoFaeC was calculated and used to predict the orientation upon immobilization in order to relate the surface properties to changes in enzyme activity (Paper III). This is described in Section 4.5.

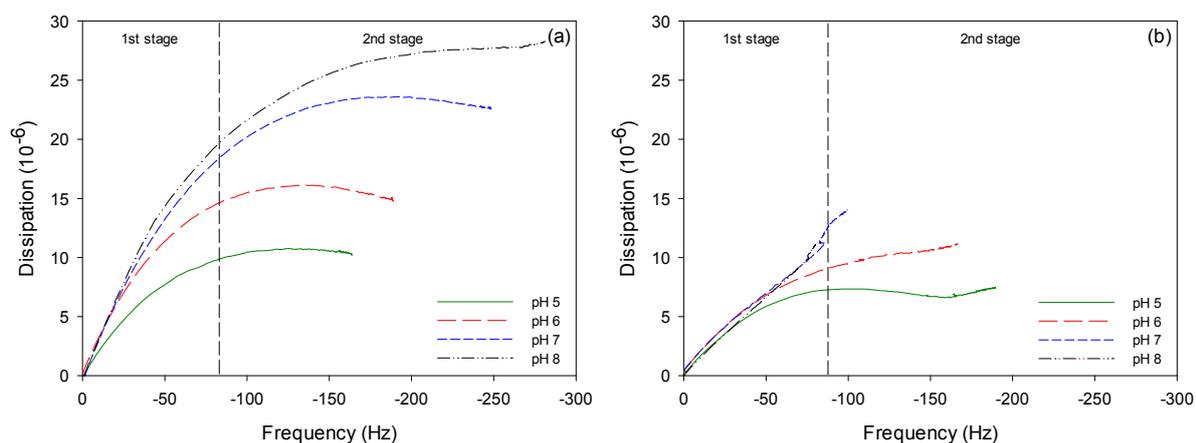


Figure 14. Dissipation as a function of frequency during the immobilization of (A) FoFaeC and (B) ROL in HMM at different pH. Higher dissipation at a certain frequency indicates a more rigid enzyme layer, and can be interpreted as more stable adsorption. (From Paper II.)

The dissipation data obtained from the QCM-D measurements during enzyme adsorption show consistency between the two enzymes FoFaeC and ROL (Figure 14). Both enzymes have low dissipation at low pH, which indicates that they have formed a rigid layer, and it can be assumed that the adsorption to the silica surface is stronger than the adsorption at higher pH. The stability measurements (washing with buffer, Figure 9) showed that the stability of the immobilization of FoFaeC as a function of pH (Figure 15) was not correlated with the pH at which the highest amount of enzyme had been immobilized (Figure 13). Rather, the stability of the immobilization was correlated with the dissipation (Figure 14A). Thus, low dissipation values obtained from QCM-D measurements indicate a rigid enzyme layer, and

can be used as a predictor of the stability of immobilization. These results are further discussed in Section 3.8, where various molecular interactions and their role during immobilization are discussed in relation to the interactions that are important for stable immobilization. The results presented in this section indicate that immobilization is highly dependent on the enzyme, and that it is difficult to predict which interactions are most influential without more information on the enzyme itself.

#### *3.8 Reusability of feruloyl esterases immobilized in mesoporous silica*

One of the main goals of enzyme immobilization is to increase the stability of the enzymes, while at the same time improving the reusability of the enzyme. If mesoporous materials are to be considered a robust immobilization support for feruloyl esterases, the stability of the immobilization must be demonstrated. Combining the results presented in Paper I and Paper II, it can be concluded that the inactivation of feruloyl esterases inside mesoporous particles is low, and that the enzymes are retained inside the particles over time in buffer solutions (Figure 15) and in 1-butanol (Figure 16). It can thus be concluded that mesoporous materials provide a robust immobilization support, with satisfactory reusability of the biocatalyst, especially compared to previous immobilization attempts with feruloyl esterases (Table 2). In comparison, it can be mentioned that cross-linked enzyme aggregates of Depol 740L (the same enzyme that was used in the study described in Paper I), showed no activity after 2 runs (6 days/run) in a 1-butanol/hexane/water microemulsion [49].

Interestingly, the condition that gave the best retention of the enzyme inside the pores, i.e. low pH (Figure 15) was not correlated with the condition that provided the highest degree of immobilization, i.e. high pH (Figure 13). Both the mesoporous particles and the flat silica surface exhibited the highest retention at low pH, while the amount of enzyme immobilized increased with pH. Based on the results obtained regarding the stability of adsorption to a flat silica surface (Figure 15), it appears that the electrostatic interactions are mainly responsible for determining whether the enzyme remains on the surface or not. The silica surface is generally expected to be net negatively charged above pH 3 [59], while it has been reported that SBA-15 has a pI of 3.8 [80]. FoFaeC, with a pI of 6.8 [77], has a net positive charge below its pI, and it is reasonable to assume that the stable interactions at lower pH are due to stronger electrostatic interactions. The same retention behavior was seen for enzymes immobilized in the mesoporous particles, although it was less distinct than for the flat surface

(Figure 15). This indicates that other forces or factors are of importance for the retention of the enzymes. One possible explanation is that the cylindrical pore wall offers more surface-protein interaction points (Figure 7C,D), which stabilize the adsorbed enzymes [81]. Another may be that enzyme molecules that desorb from the surface of the pore wall are very likely to re-adsorb on the opposite side of the wall. Enzyme molecules on the outer surface of the porous particles will probably desorb first, as easily as those from the flat surface. If the calculated values of pore filling are low, caution should be exercised when analyzing the leaching problems often reported when using mesoporous particles [55, 73], as some of the problems associated with enzyme leaching may be due to the fact that the enzyme molecules are adsorbed on the outer surface of the particles and not inside the pores.

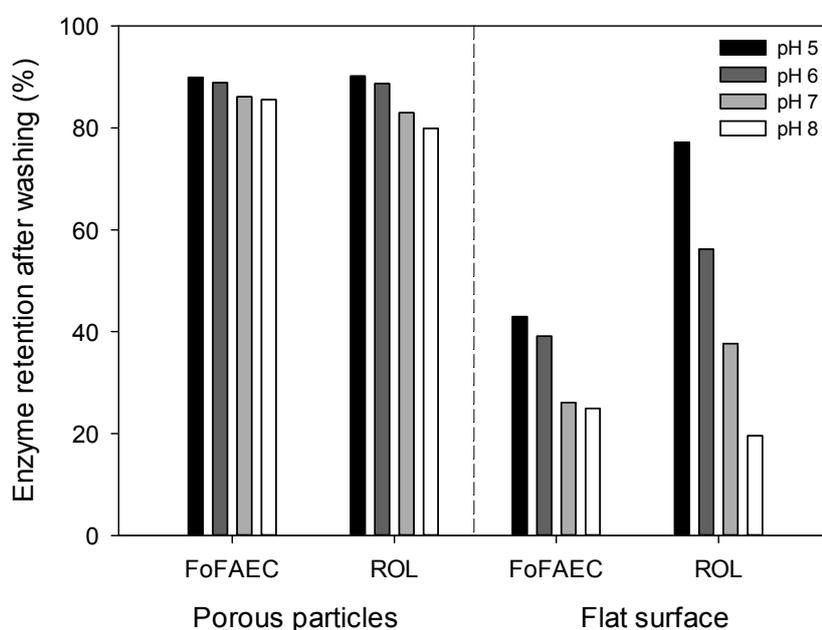


Figure 15. Stability of enzyme immobilization, expressed as enzyme retention after 40 minutes of washing with buffer, using QCM-D frequency measurements. The frequency before washing was defined as 100% and the subsequent increase in frequency was subtracted from this value. (From Paper II.)

### 3.8 Reusability of feruloyl esterases immobilized in mesoporous silica

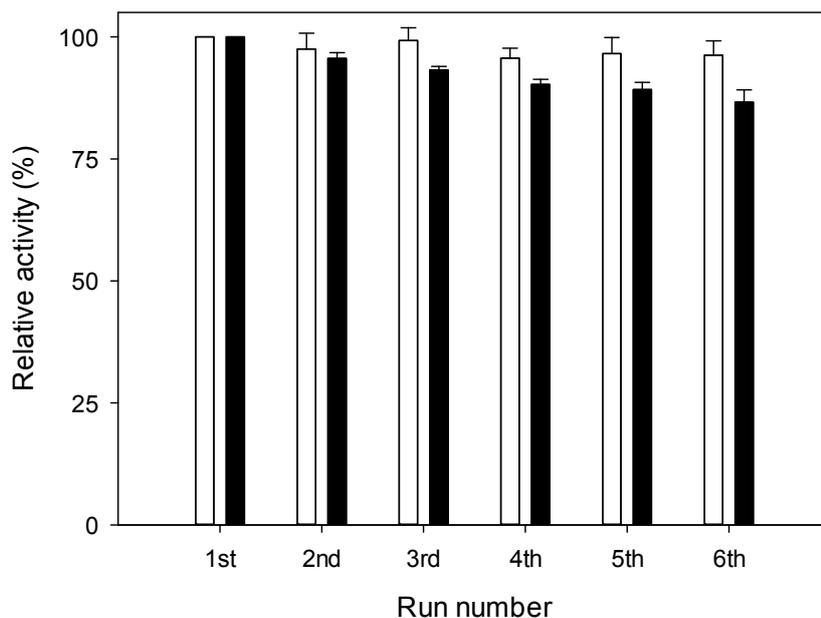


Figure 16. Retained activity of Depol 740L immobilized in SBA-15 (9 nm pore size; white bars) and non-porous silica particles (black bars) after six 6-day runs in 92.5% 1-butanol. The enzyme-containing particles were removed after each run by centrifugation, after which fresh substrate solution was added. The activity during the first run is defined as 100%. (The graph is adapted from data given in in Paper I and shows the average of 4 replicates with error bars indicating one standard deviation.)

Two completely different supporting materials were studied, with regard to particle shape and size, and pore arrangement. The more common SBA-15 particles with particle sizes of 1-2  $\mu\text{m}$ , which have a hexagonal pore alignment were used in the first study (Paper I), while HMM particles (schematically illustrated in Figure 9) with a particle size around 50 nm with randomly organized pores were used in the second study (Paper II). The feruloyl esterases investigated seemed to be stably immobilized on both these supports. There was thus no need for covalent binding or cross-linking of the enzyme, allowing the simplest possible immobilization protocol of physical adsorption.

### 3.9 *Enzyme activity must be considered to fulfill the aim of a robust biocatalyst*

The initial aim of immobilizing feruloyl esterases in mesoporous silica was to facilitate reusability of the enzyme and to demonstrate that the enzyme is stable after several runs. The results show that several factors must be considered in order to obtain satisfactory immobilization. Not only is immobilization highly dependent on the enzyme, but enzyme loading and immobilization stability are not necessarily correlated.

As discussed, pH affects both the amount of enzyme immobilized and its stability. However, the results presented in Paper III show that the pH during immobilization has a significant effect on the specific activity of the immobilized enzymes. This means that immobilization protocols should be developed and optimized in parallel by measuring the enzyme activity and studying how the enzyme activity is related to the immobilization conditions.

In this chapter, tools have been presented for the improved interpretation of immobilization results that are useful for the understanding of differences in enzyme activity following immobilization in mesoporous materials. The enzyme activity has not been discussed in this chapter, however, as will be seen in the next chapter, ignoring the enzyme activity when optimizing immobilization may lead to improved reusability of the enzyme, although it may be less active



## 4 Changes in enzymatic activity upon immobilization

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It is well known that enzyme activity can be altered by immobilization in mesoporous particles. The substrate specificity (i.e. the activity per amount or unit weight of enzyme) or selectivity of the enzymes can also be affected [53, 63]. However, what actually causes these changes is not always well understood. This is one of the main topics of Paper V, where different approaches and methodologies to bridge this gap in knowledge are discussed. The difficulty lies in the fact that different factors are often involved in altering the enzyme structure, and determining the activity of the immobilized enzyme. Immobilization conditions, pore filling, the surface properties of the support and the microenvironment inside the pores are some of these factors, in addition to pH, temperature and ionic strength, which have a more general effect on enzyme activity. If the mechanisms behind the changes in enzyme activity could be understood in greater detail, immobilization could be used as a tool to control or customize enzyme activity [63]. The most famous example is that of lipases, where a hydrophobic support material can activate the enzyme by “opening” the lid that covers the active site of the enzyme, increasing the specific activity of the enzyme compared to the free enzyme [82]. It has been shown that the activity of lipases can be controlled by carefully choosing the immobilization conditions and the type of functionalization of the support material [83].

As different amounts of enzyme were used in the experiments, it was necessary to calculate the specific activity to allow comparison with the activity of free enzymes. The transesterification yield of the product (defined as the molar amount of butyl ferulate formed compared to the initial amount of methyl ferulate, expressed as a percentage) is also discussed as a complement to the specific activity. It is also important to bear in mind that using different amounts of mesoporous particles in the reaction solution may change the viscosity of the solution, which should be avoided as the viscosity may affect both substrate diffusion and the flexibility of the enzyme configuration [84].

As the immobilization of feruloyl esterases in mesoporous materials had not been previously attempted, there was some degree of uncertainty as to whether the immobilized enzymes would be active or not. Initial experiments showed that the enzymes immobilized in the 9 nm pore size particles were active, but that their initial specific activity was 40% lower than that of free enzymes in the transesterification of methyl ferulate to butyl ferulate (Paper I).

However, the overall transesterification yields when the reactions were run for 6 days were always higher for the immobilized enzymes, exceeding 80%, whereas the yield with free enzymes was only around 50% (Paper I). In this case, measuring the initial activity is not a good indicator of the performance of the immobilized enzymes. This was also the first indication of a change in product selectivity, in that the immobilized enzymes favored transesterification over hydrolysis (see Section 4.2). This is important as not only the substrate, methyl ferulate, can be hydrolyzed, but also the product, butyl ferulate, which could explain the lower yields of free enzymes, despite their higher initial activity. It could be concluded that it was better to immobilize the feruloyl esterases in Depol 740L as this provided higher product yields, and the enzymes were firmly attached to the mesoporous particles, allowing high reusability. The yields were comparable to those reported previously (Table 2), and remained high after 6 runs.

It should be mentioned that during the course of this work, other feruloyl esterases were also studied (unpublished results). There is considerable variation in product yields between the different immobilized feruloyl esterases and in some cases almost complete inactivation of the enzyme. It can, therefore, not be assumed that all feruloyl esterases will show satisfactory activity upon immobilization. However, it may be possible to remedy this by, for example, optimizing the immobilization conditions such that the enzyme is oriented so the active site is accessible.

The enzyme activity measurements presented in this thesis are from feruloyl esterases immobilized in SBA-15 type mesoporous materials. There are however several other types of silica materials with different organization of the porous structure and particle geometry [85-87]. The oxidation activity of a cytochrome c immobilized in mesoporous silica with sheet like geometry (disordered pore size distribution and shorter length of the pores) has been shown to be 2.5 higher compared to enzymes immobilized in SBA-15 or free enzymes [88]. The higher activity was related to a higher turnover number and increased  $k_{cat}$  with an overall increase in catalytic efficiency ( $k_{cat}/K_M$ ), even though the substrate affinity was lower (higher  $K_M$ ). Interestingly, in the case of the feruloyl esterases in Depol 740L, the narrow pore size (5 nm) lowers the specific activity of the immobilized enzymes, but do not affect the product selectivity. The connection between enzyme activity and the geometry of the mesoporous material is an area where further studies are required.

Differences in activity between free and immobilized enzymes are thoroughly discussed below, as this information provides a deeper understanding of how enzymes are affected by immobilization. Changes in the activity of feruloyl esterases upon immobilization have been reported previously [34, 49]. It has also been shown that the exposure of feruloyl esterases to other solvents can alter their substrate specificity [48]. It was speculated that this was due to rearrangement of the active site. Changes in the specific activity of other enzymes have also been coupled to pore size in a study where several structural changes were detected by circular dichroism [89]. The results observed when studying immobilized feruloyl esterases led to several questions. Why are the specific activity and yields different for the immobilized enzymes? Could the immobilization conditions be influencing the enzyme activity? Differences in microenvironment are often reported in mesoporous materials, but how could these affect the immobilized feruloyl esterases?

#### *4.1 Immobilization pH determines specific enzyme activity*

The fact that the amount of immobilized feruloyl esterases is highly affected by pH was described in Section 3.7. It is also known that the pH in the reaction solution affects enzyme activity in general. The pH optima for hydrolysis and transesterification of hydroxycinnamic acids have previously been shown to be different for a chlorogenate hydrolase [90]. Therefore, pH could be used to control the product selectivity, changing towards transesterification when lowering the pH. In this section, it will also be shown that the specific enzyme activity is also determined by the immobilization pH. After immobilizing the pure feruloyl esterase, FoFaeC, at pH 5-8, the transesterification activity was compared to that of free enzymes in the same buffers (Figure 17A). The optimum reaction pH for the immobilized enzymes was shifted towards pH 6, with a clearly lower activity at pH 7-8. To separate the effect of reaction pH from the effect of pH during immobilization, the enzymes immobilized at different pH were all tested for transesterification at pH 6. Unexpectedly, the enzymes immobilized at pH 4 and pH 8 showed no improvement in activity when run at pH 6 (Figure 17B), which indicates some kind of memory effect from immobilization. It is probable that the enzymes adsorb onto the support with different strengths, as the strength of the enzyme-support interactions varies with pH. Once adsorbed, the enzyme structure may be stabilized in different ways. Other researchers have also reported immobilization conditions to be important for the final enzyme structure, especially if the enzymes are used in organic solvents with low water contents [91, 92].

#### 4.1 Immobilization pH determines specific enzyme activity

The immobilization process can, thus, not be optimized without considering the enzyme activity. Selecting the appropriate immobilization pH is therefore of greater importance than using the correct reaction pH. If pH 8 is used for immobilization, twice the amount of enzyme will be immobilized as at pH 6, but the overall yield will be lower due to the considerable decrease in specific activity, which means that it is better to immobilize the enzymes at pH 6 or pH 7 to obtain higher yields. The choice of buffer can also be used to control the transesterification activity. The specific activity was almost 3 times higher at pH 6 when the buffer was changed from citrate phosphate to 3-morpholinopropane-1-sulfonic acid (MOPS) buffer. To better understand the structure-activity relationship of the immobilized enzymes, the structure of FoFaeC was modeled. Using this model, the charge distribution on the enzyme surface could be evaluated in relation to the measured specific activities, and the active site could be characterized, allowing studies on the docking between the substrate and the MOPS molecule (as discussed in Section 4.5).

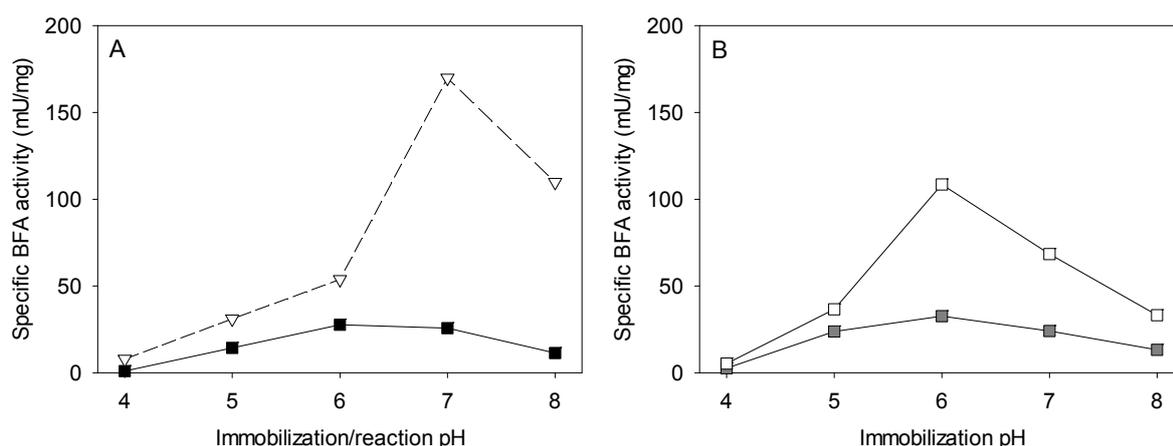


Figure 17. Transesterification of methyl ferulate with 1-butanol (92.5% v/v) into butyl ferulate (BFA) using: (A) FoFaeC (■) immobilized at the same pH as that used during the enzyme reaction, and the free enzyme (▽) at the corresponding pH (buffered with citrate phosphate buffer). (B) FoFaeC immobilized at different pH values in citrate phosphate buffer, and the transesterification at pH 6 in citrate phosphate buffer (□) and MOPS buffer (■). (Adapted from Paper III.)

#### 4.2 Changes in product selectivity towards transesterification

Apart from changing the specific activity, immobilization can alter both the substrate specificity and the product selectivity of enzymes [63]. In the case of hydrolases, there will be two possible products from synthetic reactions, as there will always be some degree of hydrolysis of methyl ferulate into ferulic acid (Figure 18). The analysis of the results presented in Paper I showed that the immobilized enzymes generally seemed to exhibit a lower degree of hydrolysis, discussed here in terms of a change in product selectivity. The fraction of the hydrolysis product, ferulic acid, was reduced and the enzymes instead favored the transesterification product, butyl ferulate, as illustrated in Figure 19. At the same time, this allowed for higher overall transesterification yields. The previously reported examples of immobilized feruloyl esterases catalyzing transesterification reactions do not state the extent to which ferulic acid or sinapic acid was generated in the reactions (Table 2; [34, 49]). Therefore, it is difficult to determine whether the immobilization of feruloyl esterases through the formation of cross-linked enzyme aggregates showed a similar shift in product selectivity. Hence, it is suggested that the formation of the corresponding acids should always be reported when hydroxycinnamic esters are used as substrate. This would allow better interpretation of the results, as a low transesterification yield could be caused by either increased hydrolysis or an overall decrease in the specific activity of the enzyme due to immobilization.

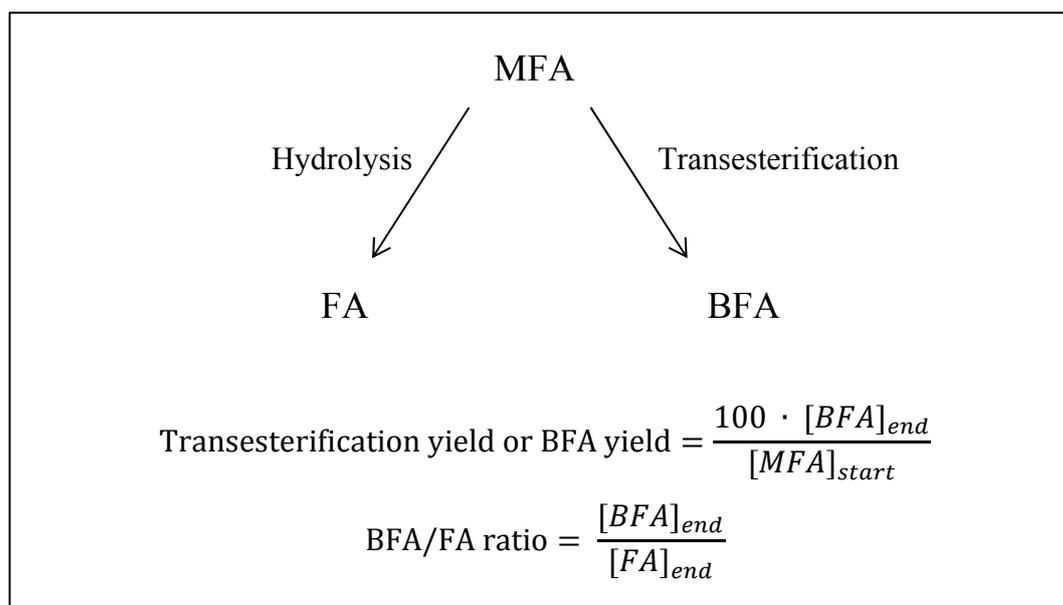


Figure 18. Definition of the different terms discussed regarding the change in activity of immobilized feruloyl esterases. The BFA/FA ratio is the ratio between the concentration of butyl ferulate and ferulic acid.

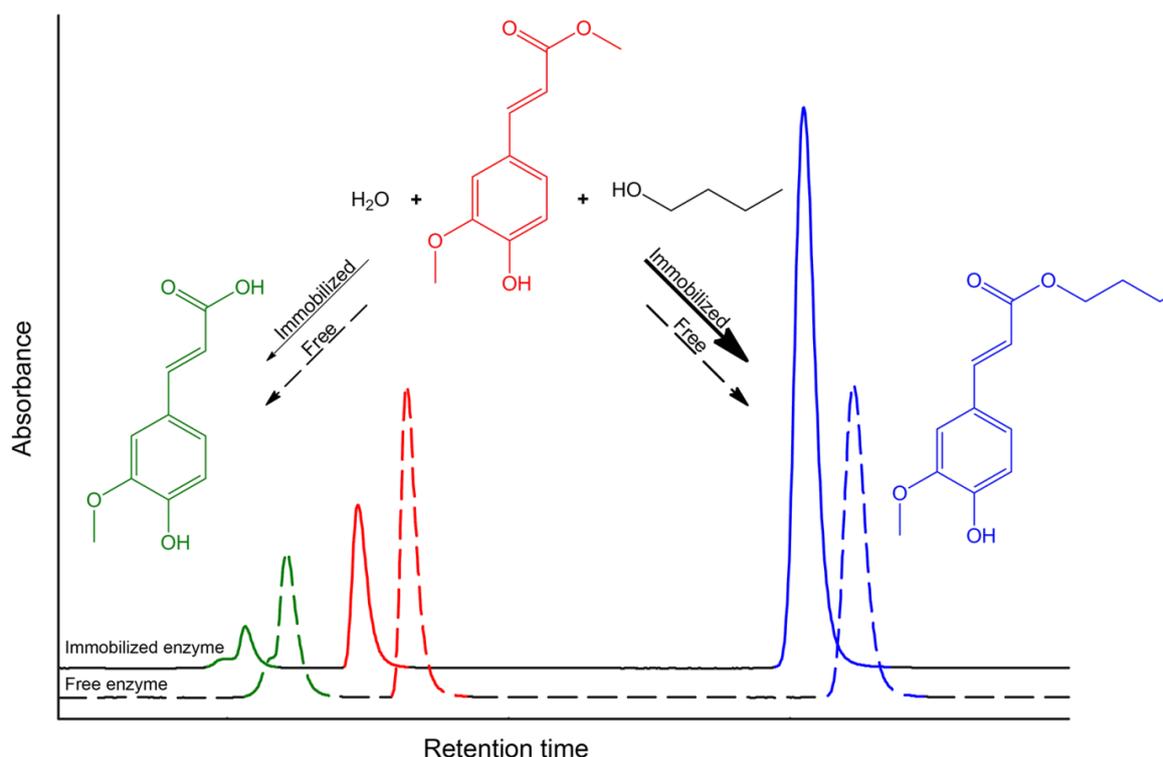


Figure 19. The change in transesterification yield compared to hydrolysis yield schematically represented by HPLC graphs for free and immobilized Depol 740L. The colors represent the substrate and products as follows: (green) ferulic acid, (red) methyl ferulate and (blue) butyl ferulate. (Adapted from the graphical abstract of Paper I.)

It is clear that there is a change in product selectivity upon immobilization, but the question is which factors cause this change. To establish whether the change in selectivity was associated with the enzyme being adsorbed inside the pores, enzymes immobilized on non-porous silica particles were studied, showing the same trend as for the mesoporous particles. The enzymes immobilized on mesoporous particles with a 5 nm pore size (assumed to be too small for the enzymes to enter) also showed the same results. Thus, the observed behavior is not a direct result of the enzyme being adsorbed in the pores.

The water content must be as low as possible, to avoid the hydrolytic activity of feruloyl esterases [30]. The water content must be set so as to obtain a compromise between keeping the enzyme active, while restricting the hydrolysis activity. At low water contents a higher BFA/FA ratio (i.e., the ratio between the concentration of butyl ferulate and ferulic acid) would be expected, manifested as an increase in transesterification relative to hydrolysis (Figure 20). However, the overall transesterification yield is close to zero at water contents from 0 to 2.5% (Paper I), while free enzyme shows a relatively high yield at a water content

of 1%. The examples of previous synthetic reactions with feruloyl esterases using cross-linked enzyme aggregates were almost exclusively performed in microemulsions with a water content around 1-3% [30, 34, 49], which correspond to the results of the present work obtained with free enzyme that increase the synthetic activity at these water contents. Free feruloyl esterases performing hydrophilic esterification of ferulic acid with diglycerol at water contents above 10% have previously been shown [47]. Using mesoporous silica as the immobilization support allows high reaction yields also at higher water contents, which reduces the amount of organic solvents needed. Additionally, the choice of reaction solution components becomes more flexible, as it is not necessary to form a microemulsion. Interestingly, the BFA/FA ratio varies between free and immobilized enzyme but has a similar profile as a function of the water content (Figure 20), but the ratios for the immobilized enzymes are factors of 5-9 higher than for the free enzymes. Furthermore, the difference in the BFA/FA ratio suggests that the availability of water, or hydration of the enzyme, is different when the enzyme is immobilized in a mesoporous silica structure.

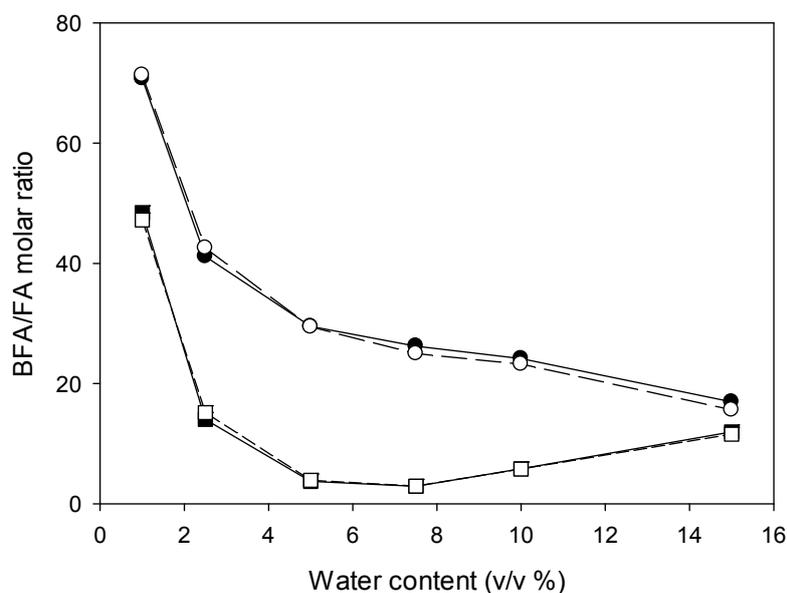


Figure 20. BFA/FA molar ratio as a function of water content for Depol 740L immobilized in SBA-15 of two different pore sizes: (●) 5 nm, (○) 9 nm; and as free enzyme sampled at two different time points (■) 46 h and (□) 168 h during the reaction. (From Paper I.)

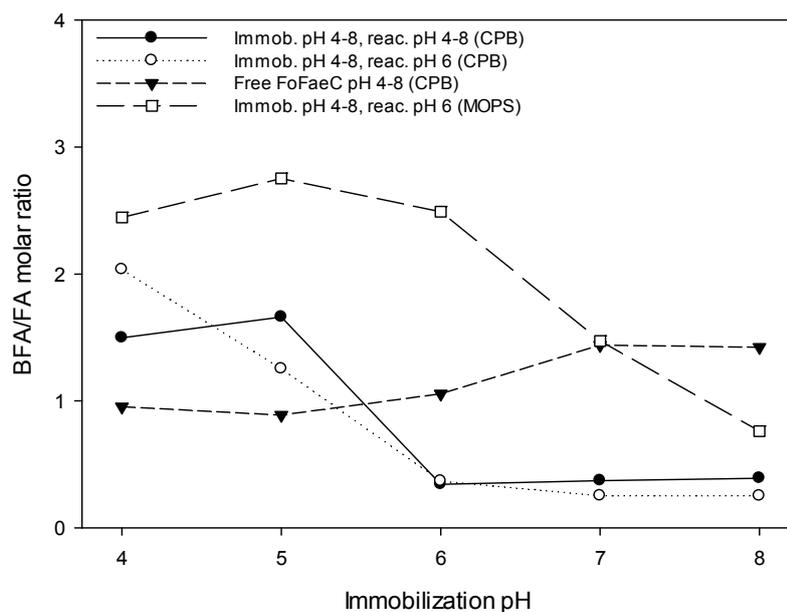


Figure 21. BFA/FA molar ratio for FoFaeC immobilized at different pH, and transesterification reactions run at the same or different pH. The enzyme was immobilized at different pH in citrate phosphate buffer (CPB) while the transesterification reactions were as follows: (●) the same pH as immobilization, (○) at pH 6 in CPB, and (□) at pH 6 in MOPS buffer. (BFA/FA ratios were calculated using the data presented in Figures 2 and 3 in Paper III.)

To investigate whether the water content inside the pores was different from that in the bulk solution, a Karl-Fischer titration was performed on the supernatant after the addition of the mesoporous particles (Paper IV). The measurements showed no difference in water content before and after the addition of mesoporous particles, indicating that the overall water content inside the pores was probably the same as in the surrounding bulk solution. It has been suggested that the organization of water and butanol molecules may be different inside the pores [93]. Another factor that can affect enzyme activity is the solubility of the substrate and product. Differences in solubility have been shown to affect the activity of lipases used for the esterification of hydroxycinnamic acids, where a low substrate solubility relative to the product solubility was found to be beneficial for the overall product yield [37]. It can be speculated that the solubilities of ferulic acid, methyl ferulate and butyl ferulate are different in the microenvironment close to the silica surface from that in the bulk solution. This difference in solubility depending on the microenvironment is further discussed in Section 4.4 and in Paper V.

The difference in BFA/FA ratio between immobilized and free enzyme is maintained when using pure FoFaeC (Paper III) instead of the crude feruloyl esterase preparation Depol 740L (Paper I). However, the ratios are different in absolute terms, which shows that the effect of immobilization is enzyme dependent (the BFA/FA ratio for Depol 740L is  $\approx 9$  and for FoFaeC  $\approx 4$ , at a water content of 7.5% buffered with MOPS). It is also reasonable to assume that structural changes in the enzyme are not the only cause of the shift in product selectivity, as it is improbable that similar changes would be induced in enzyme structure in the two different feruloyl esterases by immobilization.

Immobilization at different pH also had a considerable effect on the product selectivity of FoFaeC (Figure 21). The pH during immobilization seems to have a greater influence than the pH during the enzyme reaction. Hence, both specific activity (see Figure 17) and selectivity are influenced by the immobilization pH. Again, these results show that the choice of immobilization conditions will be a tradeoff between improving transesterification and maintaining the overall yield.

MOPS buffer generally led to both higher yields and a larger fraction of butyl ferulate than reactions run in citrate phosphate buffer (Figure 21). To further study the dependence of product selectivity on the buffer, two other buffers, MES and PIPES, were also investigated (unpublished results). These buffers have structural similarities with MOPS (Figure 22), and have also been shown to be useful in biological systems [94]. It was found that the choice of buffer greatly influenced the selectivity of the enzyme (Table 6). However, it is difficult to draw any direct conclusions related to the properties of the buffer molecules. Nevertheless, it can be speculated that the buffer molecules interact with the support surface in different ways, possibly shielding the surface charge to different extents. Differences in charge density at the surface may affect the enzyme during immobilization, which in turn could change the orientation of the enzyme molecule or give rise to structural changes. Additionally, the buffer molecule itself may interact with the enzyme. This hypothesis was tested through a study of the docking between MOPS and a model structure of FoFaeC, as described in Section 4.5.

## 4.2 Changes in product selectivity towards transesterification

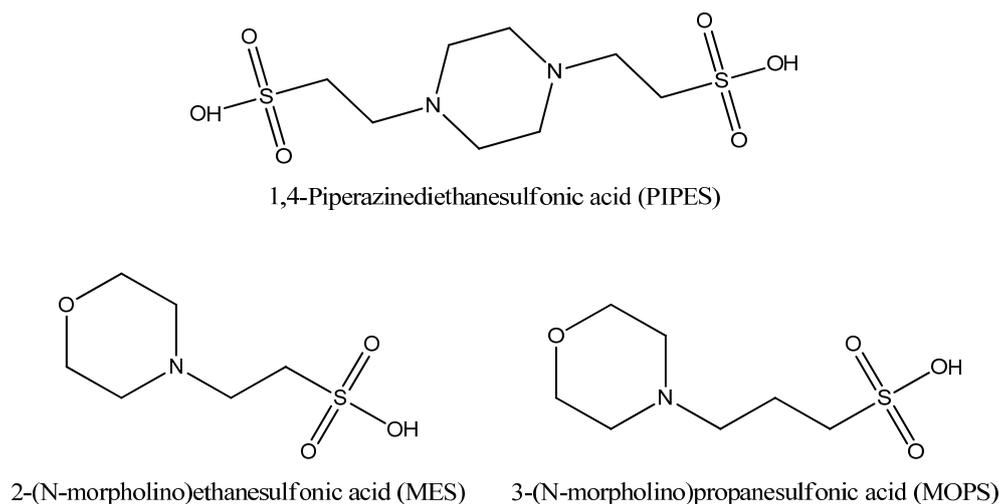


Figure 22. Molecular structures of the three buffers: PIPES, MES and MOPS.

Table 6. BFA/FA molar ratios in reactions with free and immobilized FoFaeC in different buffers. Immobilization was performed in the same buffer as that used for the enzyme reactions.

Buffer	BFA/FA molar ratio	
	Immobilized	Free enzyme
MES <sup>a</sup>	2.4	0.9
PIPES <sup>a</sup>	4.6	2.5
MOPS <sup>a</sup>	4.3	1.2
Citrate phosphate buffer <sup>b</sup>	0.3	1.1

<sup>a</sup>Unpublished data

<sup>b</sup>Data taken from Figure 21.

An additional explanation could be local differences in pH inside the pores, which could influence the selectivity. The microenvironment was studied in terms of apparent pH by fluorescent labeling of the enzyme with a pH-sensitive probe to determine the extent to which the enzymes experience a different pH compared to free enzymes (Paper IV). Studying functionalized mesoporous silica (e.g. hydrophobization) or other types of materials (e.g. alumina or titanium) could provide additional information on the material properties that are important for the selectivity of feruloyl esterases.

The various factors influencing the BFA/FA ratio are summarized in (Table 7). Based on the results of these studies, it is not possible to determine conclusively what causes the shift in product selectivity of feruloyl esterases upon immobilization. However, the solvent dynamics in the microenvironment close to the silica surface seem to be important, as are the immobilization pH and the choice of buffer. Therefore, the structure of FoFaeC was modeled together with the MOPS molecule to further study the effect of the structure of the buffer molecule, as discussed in Section 4.5 and Paper III. It would be interesting to investigate whether the product selectivity is similar for other reactions with other substrates, for example, the saccharification of other hydroxycinnamic acid esters. Other mesoporous materials, based on for example alumina, titania or carbon, should also be studied. These materials vary in the composition of their molecular surface, which means that both the hydrophobicity and isoelectric point of the surfaces will be different. Gaining better knowledge on which interactions influence the product selectivity of feruloyl esterases is the key to high transesterification yields and the reduction of side product formation through hydrolysis.

Table 7. Summary of the factors that influence product selectivity, defined as the BFA/FA ratio, in comparison with the BFA/FA ratio of free feruloyl esterases in solution

<b>Factor/property</b>	<b>Effect on BFA/FA ratio</b>	<b>Comments</b>
Water content	Increases with decreasing water content.	Similar BFA/FA vs. water profiles for free and immobilized enzyme, but a generally higher ratio for immobilized enzyme.
pH	Increases with decreasing immobilization pH.	Both the pH during immobilization and the pH during the enzyme reaction influence the ratio.
Buffer	MOPS and PIPES buffer give the highest ratios.	The choice of buffer has a considerable influence. Structural similarity of the buffer molecule and the substrate molecule seems favorable.
Silica surface	Both porous and non-porous silica surfaces increase the ratio.	The effect on the ratio is due to the enzymes being close to a silica surface, rather than being confined in the pores.
Pore diameter	Minor effect on the ratio.	The charged silica surface and the micro-environment close to the surface have a greater influence than the porous structure.
Enzyme	Varies for different feruloyl esterases.	The ratio is highly enzyme specific, but is generally higher for immobilized feruloyl esterases.

*4.3 Studies of local pH inside the pores using a fluorescent enzyme-linked probe*

It is known that the microenvironment close to surfaces, and thus also inside porous structures, is different from that in the surrounding bulk solution [55, 63]. This means that immobilized enzyme molecules experience different local reaction conditions at their immobilization site [63]. A change in the specific activity upon immobilization may therefore not necessarily be related to structural changes of the enzyme, but could be explained by the microenvironment. It is less clear how the microenvironment differs from that in the bulk solution, and how these differences can be studied.

The amount of immobilized feruloyl esterases in mesoporous silica was found to be highly influenced by the choice of pH during immobilization (Paper III). The specific activity and product selectivity of the enzymes are also influenced by pH during both immobilization and the enzyme reactions (as shown in Figures 17 and 21, respectively). Based on these results it was hypothesized that the pH inside the pores could be different from that in the bulk, which would mean that the enzymes experienced a different pH from that set by the buffer. In order to test this hypothesis, a protocol was developed for labeling enzymes with a fluorescent probe (Paper IV). Briefly, the probe was attached to the enzyme (FoFaeC) with a reactive succinimidyl ester derivative of Seminaaphtharhodafuor-1 (SNARF-1) prior to immobilization in the mesoporous particles (Figure 23). A reference curve was then created based on the response of free SNARF-1-labeled FoFaeC in solution (Figure 24A). The apparent pH inside the pores could then be determined (Figure 24B).

Previous attempts to study local differences in pH in mesoporous materials using other pH-sensitive fluorescent probes have shown variation in the pH inside the pores, indicating neutral or weak acidic conditions [95]. However, the approach used in that study required surface functionalization of the mesoporous silica, which significantly altered the surface properties of the material, and thus also the microenvironment at the surface. This would probably also affect the immobilization behavior of the enzymes as both the hydrophobicity and charge density at the surface would be changed. The method using SNARF-1 to label the enzyme, on the other hand, leaves the material itself unchanged, and the local pH is only measured at the sites where the enzyme molecules are located. This method is thus more suitable for studying applications involving immobilized enzymes.

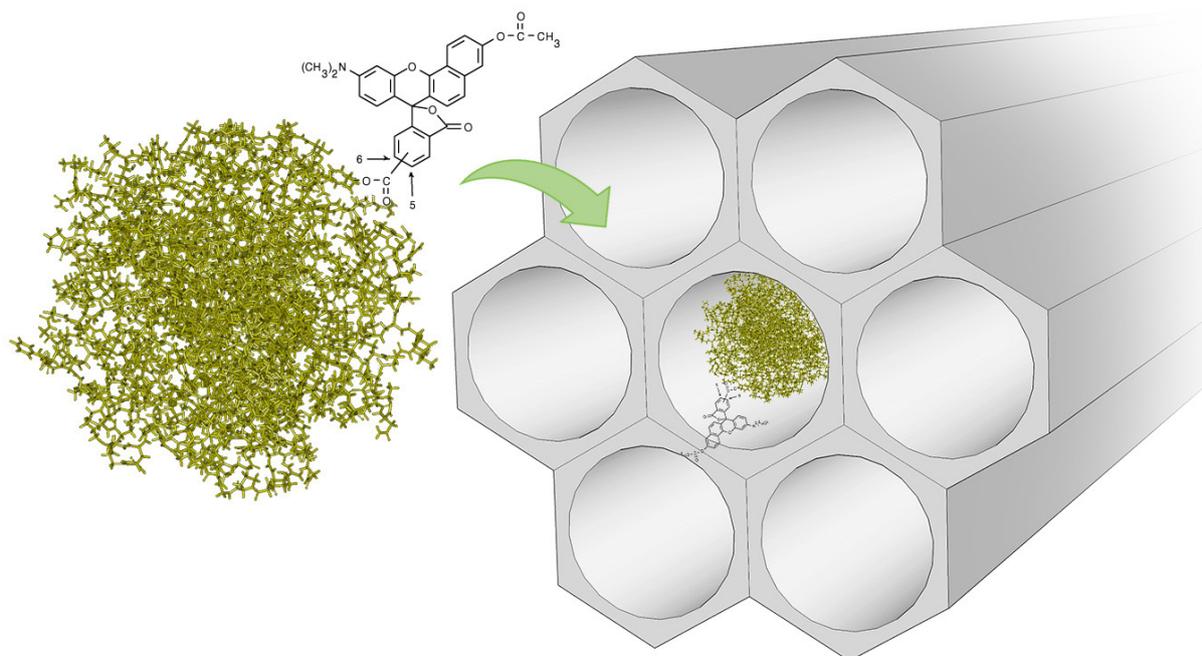


Figure 23. Binding of SNARF1 to FoFaeC prior to immobilization in mesoporous silica. The figure also illustrates the requirement that the pore size (typically 8-9 nm) should match, or be slightly larger than, the dimensions of the enzyme.

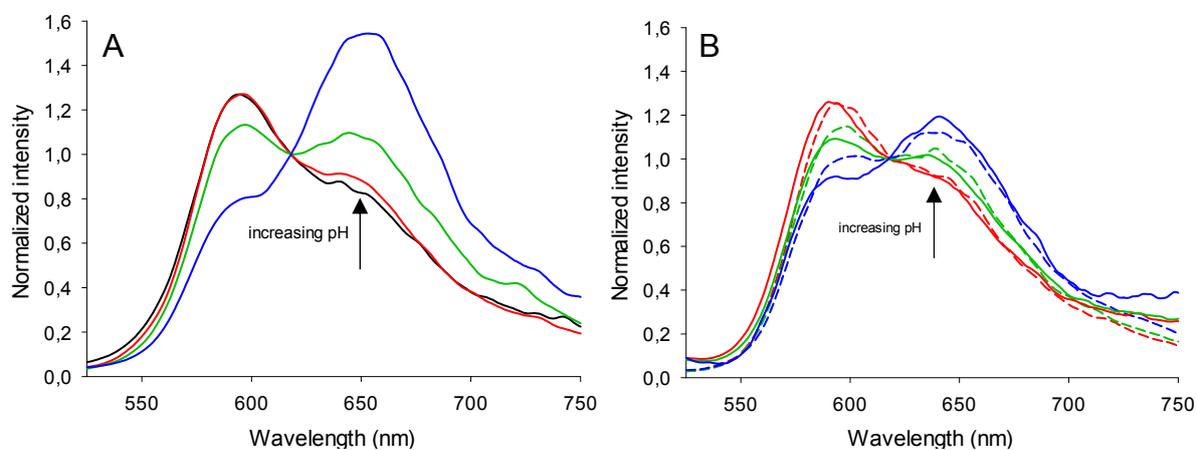


Figure 24. (A) Fluorescence emission spectra from free SNARF-1-labeled FoFaeC free in citrate phosphate buffer at different pH 5 (black); 6 (red); 7 (green) and 8 (blue). (B) Fluorescence emission spectra from SNARF-1-labeled FoFaeC immobilized in SBA-15 in citrate phosphate buffer (solid lines) or in 92.5/7.5% 1-butanol/buffer mixtures (dashed lines) at different pH. The spectra were normalized at 618 nm. (From Paper IV.)

To determine whether the probe had any direct effects on the enzyme itself, the enzyme activity of SNARF-1-labeled FoFaeC was measured (Paper IV, Supplementary material). The activity of FoFaeC was not affected by the attachment of SNARF-1 and, therefore, it is likely that the overall tertiary structure is maintained. Furthermore, the fluorescence spectra obtained with SNARF-1-labeled FoFaeC are similar to those from SNARF-1-labeled bovine serum albumin (BSA), used as a standard protein and control in setting up the method. This indicates the fluorescence response of the probe has little dependence on the enzyme. This methodology will probably also be suitable for studying the local pH when other enzymes are immobilized in other support materials, assuming that the material does not quench or interfere with the fluorescence. The sensitivity of the method allows the detection of at least 100 times lower protein concentrations than used in the present experiments, which further increases the applicability of the method. It is also reasonable to assume that the protocol for labeling enzymes prior to the immobilization can be generalized to other enzymes.

#### *4.4 Shift of the fluorescence spectra of SNARF-1 in butanol*

The results obtained by labeling FoFaeC with SNARF-1 in different buffer solutions indicate that the mesoporous material SBA-15 has a buffering effect towards neutral pH (compare Figure 24A with B). Based on a titration curve for SNARF-1-labeled BSA (Paper IV), it was found that at pH 6 in the buffer, the pH in the pores was 6.4, and at pH 8 in the buffer, the pH in the pores was 7.7. This indicates that the pH in the microenvironment of the enzymes in the pores is different from that in the bulk solution, although the difference is small.

The response of the probe in the reaction mixture (92.5/7.5% 1-butanol/buffer) is very similar to that in the solution containing only buffer (Figure 24B). This could be an indication of a high water content inside the pores as the pH values were similar. However, the Karl-Fischer titrations, mentioned in Section 4.2, showed that the overall composition of the solution in the pores should be the same as in the bulk solution. The spectra obtained from free labeled FoFaeC and BSA in the same 1-butanol/buffer solution were distinctly different from those obtained from immobilized enzymes, indicating an acid environment between pH 6 and 8 (Figure 25). The shift in the spectra is independent of the presence of the enzyme, as free SNARF-1 (not attached to an enzyme) exhibited the same behavior. Spectral shifts of SNARF-1 have been reported previously in organic solvents including 1-butanol [96]. The results of these studies show that the environment inside the pores is quite different from that

in the bulk, but further studies are required to obtain a more detailed understanding of how the microenvironment affects enzyme activity.

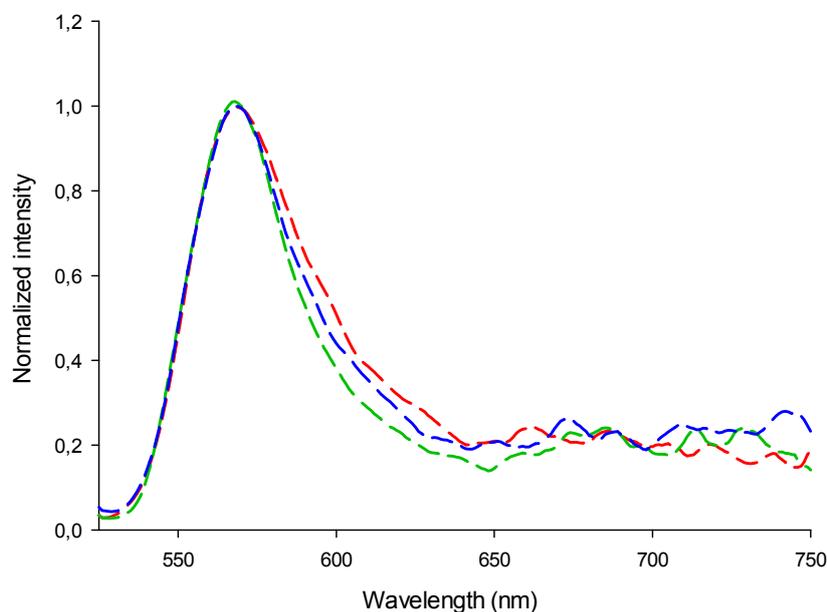


Figure 25. Emission spectra from free SNARF-1-labeled BSA in 92.5% 1-butanol and 7.5% citrate-phosphate buffer at different pH 6 (red); 7 (green), and 8 (blue). The spectra were normalized at 570 nm. SNARF-1-labeled FoFaeC showed the same response. (From Paper IV.)

The difference in product selectivity upon immobilization of feruloyl esterases can probably also be linked to the different microenvironment. The viscosity and density of the solution, substrate diffusion and solubility, and the orientation and dynamic organization of molecules are some of the factors directly affected by changes in the microenvironment, and which may alter enzyme activity [97]. Product solubility should also be taken into account, as low product solubility has been posed as an explanation to low yields for the formation of feruloyl glycerol [28]. Finding a solution composition that allows high solubility of both the product and the substrate may therefore be necessary. Additionally, layering of molecules and the arrangement of alcohols inside the pores can create concentration gradients [93, 97].

The transesterification of methyl ferulate to butyl ferulate is highly water dependent. However, at water contents  $\geq 5\%$ , the transesterification yields are relatively stable for immobilized feruloyl esterases (Paper I). This indicates that the environment inside the pores, or in the proximity of the silica surface, is more controlled, i.e., the enzyme in this environment is not as responsive to changes pH in the bulk solution as free enzymes, which show varying

yields at the same water contents. It can be speculated that the small buffering effect of the material on the pH could also be due to differences in the organization of the molecules inside the pores. Based on the same reasoning, the increased stability of enzymes immobilized in mesoporous materials reported previously [59, 73], could be the result of a more stable environment inside the pores in terms of pH and water. This differs from the common perception that greater enzyme stability is due to an increase in the number of interaction points between the enzyme molecule and the support material. Changes in enzyme activity have also been linked to different degrees of hydration of the enzymes inside confined spaces [98, 99].

Attempts have been made by others in our research group to develop fluorescent probes whose spectra change depending on the polarity of the solution, in order to provide complementary information to that obtained with the pH probe (unpublished results). This would be especially useful in determining whether the enzymes experience a more hydrophilic or hydrophobic environment inside the pores compared to the bulk solution. However, these attempts have so far not been successful due to difficulties in creating a standard curve based on probe spectra obtained from a range of solutions with different polarity. The synthetic performance of hydrolases (i.e., avoiding hydrolysis) depends to a large extent on the equilibrium concentrations of the substrate and products [100]. Information on the apparent polarity inside the pores would allow a better understanding of the causes of differences in enzyme activity, as well as aiding in the prediction of substrate solubility and partitioning. Several methods of characterizing the microenvironment are reviewed In Paper V. No single approach can be used to determine the solvent composition inside the pores. Therefore, a combination of different methods will be necessary to obtain a more complete understanding of the microenvironment inside mesoporous materials. The density of silanol groups on the surface, and the hydration of enzymes and support surfaces are examples of factors that require further study.

#### 4.5 *Modeling of the enzyme structure*

Enzyme activity is closely related to its structure, and the interactions between the enzyme and the immobilization support may induce changes in the structure that could alter the specific activity or selectivity of the enzymes. If changes in enzyme activity upon immobilization are to be understood in greater detail, analysis of the enzyme structure is necessary.

The structure of FoFaeC was modeled with the I-TASSER structure predictor, which uses multi-template modeling and alignment of secondary structures based on homology with known structures in the protein database (PDB) (Paper III). The location of the active site was determined by performing studies on the docking between the substrate, methyl ferulate, and FoFaeC. The surface charges were also calculated at different pH (Figure 26). At pH 4, positively charged patches were found to dominate close to the entrance of the active site, which is probably the site of immobilization, as the silica surface is negatively charged (pI 3.0-3.8 [59, 80, 101]). Blocking of the entrance to the active site could thus explain the almost complete inactivation of the enzymes immobilized at pH 4 (Figure 17). At higher pH, the orientation of FoFaeC upon immobilization is less predictable as the distribution of negative and positive charges is more random.

As discussed in Section 4.2, the product selectivity is buffer dependent, and many studies on feruloyl esterases have employed MOPS as buffer, at a pH close to neutral. It had been hypothesized that the similar structures of MOPS (Figure 22) and hydroxycinnamic acids (Figure 2), could allow MOPS to interact with the enzyme. Therefore, the docking of MOPS in the active site of the enzyme was studied using the model structure of FoFaeC (Paper III). An interaction was seen with the active site residues, although with a lower affinity, and it was speculated that these interactions could induce structural changes in the enzyme. Further modeling of the enzyme structure showed differences in the backbone structure in the presence of MOPS. It would be interesting to model the structure in the presence of the other buffers used in this work (MES and PIPES) to expand our knowledge of how the structure of the buffer molecule affects the structure of FoFaeC, and whether the same effect can be expected on other feruloyl esterases.

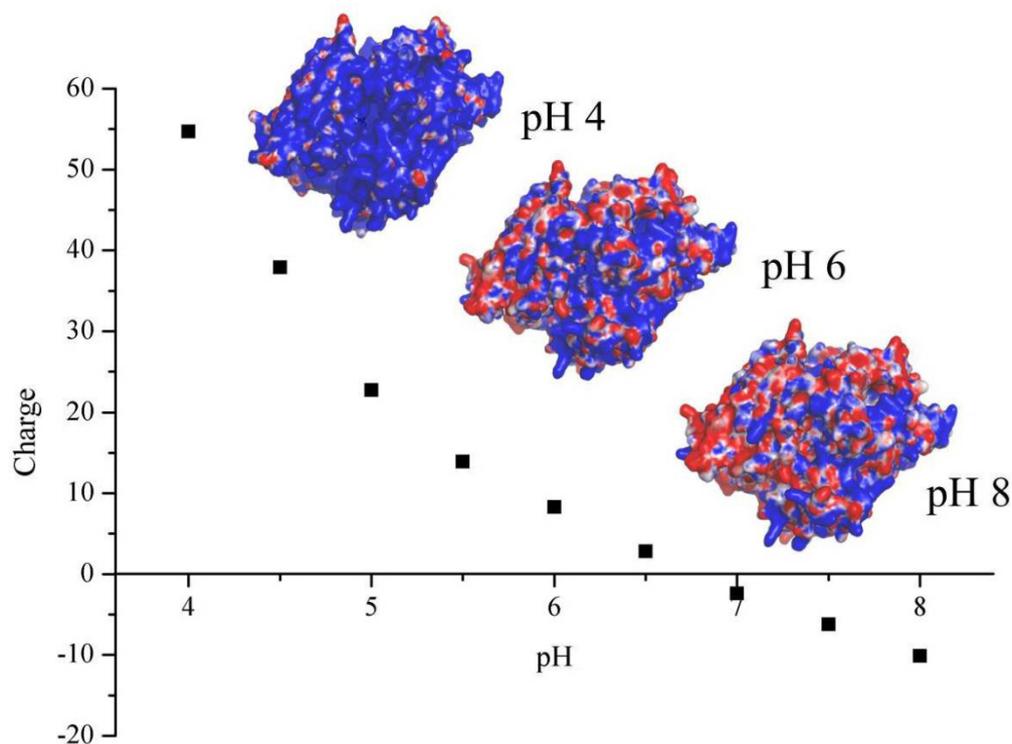


Figure 26. Net theoretical surface charge of FoFaeC as a function of pH. Negatively charged amino acids are depicted red and positively charged ones blue. (From Paper III.)

Knowledge of the enzyme structure provides additional knowledge on how the activity is affected by immobilization that cannot be obtained through experimental studies alone. Models can never fully explain reality, and must to some extent be applied in the light of experimental results. Although they may be imperfect, models still provide insights into the mechanisms behind altered enzyme activity that cannot be obtained with other approaches. The next step in the modeling of enzyme structures is to include a porous silica structure. This would allow modeling of the adsorption process, and structural changes in the enzyme and its orientation to be accurately predicted. Changes in specific activity or selectivity could also be explained in detail when structural changes upon adsorption to the surface are taken into consideration. Attempts have been made to model the adsorption of proteins to on a flat silica surface [102], and to model proteins in confined spaces [103]. Models of porous nanostructures have been developed and used in molecular dynamics simulations [103-105]. However, these models must be adapted to better resemble mesoporous materials with a defined silanol density on the surface.

Simulations of porous structures generally lack experimental validation, which is necessary for the correct interpretation of the models. Paper V provides a detailed discussion on this

topic, describing which methods can be applied to study how the interactions between enzymes and the support material can bring about structural changes in the enzyme itself. Examples of techniques, complementary to simulations of molecular dynamics, which can give further insights into structural changes upon immobilization, are circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR) and tryptophan fluorescence measurements. FTIR and variants of nuclear magnetic resonance (NMR) and X-ray photoelectron spectroscopy could also be used to study the surface of the support, and to determine which protein-surface interactions take place during immobilization.



## 5 Conclusions

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Immobilization of feruloyl esterases in a mesoporous support material provides a biocatalyst with excellent reusability, allowing high yields (>80%) in the modification of methyl ferulate over at least six 6-day runs in a butanol solution. This fulfills the aims set out in Paper I and validates the initial hypothesis that immobilization in mesoporous silica would solve the problems associated with enzyme inactivation and poor reusability reported previously. Mesoporous silica is evidently a robust immobilization support for feruloyl esterases, especially considering that the simplest possible immobilization technique, physical adsorption, could be used. This approach is both practical and economical for industrial applications, as no functionalization of the material nor cross-linking of the enzyme is necessary.

The aim of the study described in Paper II, was to gain a deeper understanding of the immobilization process through the development of a method that allows this process to be studied in real time. The results obtained with QCM-D revealed that the immobilization process consisted of two stages: initial covering of the outer surface of the particles, before the pores are filled. This study also demonstrated the importance of confirming that most of the enzyme is immobilized inside the pores when interpreting the results. Paper V presents a new way of calculating the extent to which the pores are actually filled with enzymes (pore filling). This allows the comparison of enzyme immobilization in different mesoporous materials, and makes the interpretation of the results more relevant as they are closely linked to the utilization of the pore volume, rather than the weight of the support material. The calculation of pore filling from published data can also be used as mathematical proof of enzyme immobilization inside the pores. However, there is still a need to define the degree of pore filling at which the amount of enzyme on the outer surface can be neglected compared to the amount inside the pores. In an ideal case, the fraction of enzyme molecules inside the pores could be defined.

The dependence of the amount of enzyme immobilized on pH, is surprisingly not correlated with stability once the enzyme is adsorbed on the pore wall. The stability of immobilization is probably determined by the degree of electrostatic interaction, while the interactions causing the enzyme to enter the pores are more likely to be hydrophobic and less strictly related to attractive electrostatic interactions between the enzyme and the surface.

The change in product selectivity towards transesterification and the change in optimal pH show that immobilization can be used as a means of controlling enzyme activity. The overall higher transesterification yields of immobilized feruloyl esterases compensate for the general decrease in initial specific activity. The pH during immobilization has a considerable influence on the activity, and measuring activity of enzyme immobilized at one pH is, therefore, not sufficient. The electrostatic interaction can be strong enough to inactivate the enzymes through structural changes when immobilization is performed above the isoelectric point of the material (negative net charge), but below that of the enzyme (positive net charge). The aim of the study presented in Paper III was to explain the effect of immobilization pH on the enzyme activity, and to link this to the enzyme structure through modeling of the structure of FoFaeC. The results showed that the orientation of the enzyme molecules and accessibility to the active site are important in determining the specific activity of the immobilized enzyme. It was also revealed that enzyme loading must be optimized in parallel with optimization of the reaction conditions, which are likely to be different for free and immobilized enzymes.

The experimental setup for the synthetic use of feruloyl esterases can also be considered robust in terms of the composition of the reaction solution since high transesterification yields were observed for a broad range of water contents  $\geq 5\%$ , while the hydrolysis yields were low. Previous studies have usually been limited to microemulsions with water contents below 5%, which reduces the flexibility in the choice of reaction solution. The environment inside the pores appears to be more controlled in terms of solvent composition, and does not reflect the environment of free enzymes in the bulk solution. The specific transesterification activity will always be higher than the hydrolytic activity at low water contents in the reaction solution. However, lowering the water content too much will decrease the transesterification activity in absolute terms, while hydrolysis of the substrate is almost completely inhibited. Therefore, optimization of the reaction conditions is a tradeoff between avoiding hydrolysis and ensuring there is enough water for the enzyme to be sufficiently active.

The microenvironment surrounding the enzymes inside the pores is clearly different from that of the free enzymes in the bulk solution. A new approach for pH measurements inside the pores was developed involving the labeling of enzymes with a fluorescent probe (Paper IV). This method causes no modification of the support material itself, and can be generalized to any immobilization application. The hypothesis that the pH inside the pores differed from that

in the bulk solution was confirmed, as a slight buffering effect inside the pores towards neutral pH was observed. The difference in pH can probably partially explain why the pH optima for immobilized and free feruloyl esterases are different. Synthesis reactions with feruloyl esterases require careful control of the pH and water content in the reaction solution, and an understanding how these factors differ inside the pores is the key to optimizing the yield.

Much of the research presented in this thesis was focused on a particular class of enzymes, feruloyl esterases. However, the methods developed can, to a large extent, be generalized and applied to immobilization applications with other enzymes. The QCM-D method can be applied to any enzyme that adsorbs onto any type of mesoporous particles, as long as the particles themselves can be bound to the QCM-D crystal to allow detection of adsorption. Similarly, the approach of labeling enzymes with a pH-sensitive dye further expands the tools available to characterize the complex environment inside the pores.



## 6 Future perspectives

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Awareness of biocatalysts and their potential is constantly increasing, and environmental considerations are likely to force more chemical reactions to be catalyzed by enzymes in the future. This work can be seen as a step closer to making feruloyl esterases a viable option for the synthesis of a broad range of hydroxycinnamic esters with antioxidant properties. An interesting continuation of this project would be to demonstrate the ability of immobilized feruloyl esterases to catalyze reactions in a real process, followed by purification of the synthesized product. The mesoporous particles on which the enzymes are immobilized could be packed in a column for use in a continuous process, to show that high yields and good reusability can be obtained in a setup that is more commercially relevant. Applying the enzyme-containing mesoporous particles in the catalysis of reactions with other hydroxycinnamic acids is another important task that remains to be done.

Being a relatively new class of enzymes, there are still many annotated but uncharacterized feruloyl esterases. It will be necessary to explore the substrate specificity of these uncharacterized feruloyl esterases to have enzymes available that can modify the whole range of hydroxycinnamic acids with their broad diversity in subgroup substitution. Studies on the kinetics of the immobilized enzymes should also be performed. Studies on substrate affinity or catalytic efficiency can help in the interpretation of changes in enzyme activity and in understanding the mechanisms involved. It is also important to be able to distinguish between changes in enzyme structure and differences in activity resulting from the microenvironment inside the pores. This determines whether the reaction conditions should be changed to control the enzyme activity, or if the properties of the support material should be altered. Using an enzyme that is inactivated upon immobilization and finding the conditions or support material in which the enzyme is active, would probably provide more information than simply optimizing the activity of an already active enzyme.

If research on mesoporous materials as an immobilization support is to follow the direction of more rationally designed biocatalysts that show good reusability and high yields, more fundamental knowledge is required to explain the changes in enzyme activity upon immobilization. Modeling of the enzyme structure in the proximity of the support surface will be necessary. Key steps have been taken, but simulations of the molecular dynamics of an enzyme adsorbing onto a curved silica pore wall remain to be performed. In an ideal situation,

it would be possible to alter conditions such as the pH, or hydrophobically functionalize the support surface in the model, to provide accurate predictions of enzyme orientation and structural changes. These models could also give valuable insights to the mechanisms and the nature of the interactions between the enzyme and the surface at a fundamental level, which are not easily studied with existing experimental methods.

Interdisciplinary collaboration will be necessary, as obtaining the full picture of the confining effect of pores on enzymes will require a combination of physical chemistry theory and further modeling of the enzyme structure, together with the development of new characterization methods. Thermogravimetric analysis of the solvent composition inside the pores or NMR diffusometry measurements of the dynamics of molecules are two techniques that can be used to study mesoporous particles, preferably in connection with molecular dynamics simulations. In conclusion, there is still plenty of scope for studying the physical and chemical properties of the special environment in mesoporous particles that determine the function of immobilized enzymes.

The driving force causing the enzymes to enter the pores is an important research question. Finding the answer to this question would help, for example, when choosing the pore size, or whether the support material should be hydrophobically functionalized in order to promote enzyme entrance into the pores. Potential limitations on substrate diffusion inside the pores should also be investigated, as knowledge of the enzyme activity as a function of particle size (or pore length) would be useful in the optimization of material synthesis.

This thesis and the appended papers can be seen as a guide to further studies on the effects of immobilization on enzymes. It is necessary to determine the relations between immobilization conditions, the microenvironment of the pores, material properties and enzyme activity for the application of feruloyl esterases in commercial biocatalysis processes.

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